(12) EUROPEAN PATENT APPLICATION

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(54) Detection and/or identification of microorganisms in a test sample using bioluminescence or other exogenous genetically-introduced marker.

(57) A method for determining the presence of microorganisms in a test sample. Exogenous DNA containing a luminescent system or other genetic marker system derived from a suitable donor source is introduced by genetic means into a host microorganism which lacks or poorly expresses the donor DAN and whose presence it is desired to detect. Expression of the donor gene system allows the detection of the host microorganism. Compositions of bacteriophages and plasmids a well as a method for detection of antibiotics in a test sample are described.

EP 0 168 933 A2

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DETECTION AND/OR IDENTIFICATION OF MICROORGANISMS IN A TEST SAMPLE USING BIOLUMINESCENCE OR OTHER EXOGENOUS GENETICALLY-INTRODUCED MARKER

FIELD OF INVENTION

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The invention herein described concerns analytical methods, test means, compositions, and methods of preparation thereof for detecting the presence of bacteria in a test sample. Principally, the invention

10   pertains to the determination of bacteria in areas related to health care, such as in the analysis of human excretory products or body fluids, (i.e., urine, blood, feces) for the purpose of aiding diagnosis, or to detect bacterial contamination in foodstuffs. In these and other fields it is important to rapidly, accurately and economically detect the

15   presence of specific bacteria.

DESCRIPTION OF THE PRIOR ART

There are many tests for determining the presence of bacteria and

20   identifying their type. The vast majority are based on the growth of bacterial cultures isolated from sample material containing unidentified bacteria. The specie or species of bacteria that propagate themselves on a selective medium are then isolated and typed by microscopy, physiological tests, sensitivity to antibiotics, uptake of various stains,

25   serology, etc. Many biological materials, such as for example food or skin, contain many kinds of bacteria. Most such organisms are not of central interest in respect to the aim of the tests (e.g., toxin production, pathogens, etc.). Therefore, most tests employ media that are as selective as possible and which allow the growth of bacteria whose

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presence is to be determined while trying to prevent the growth of as many as possible of those kinds of bacteria that are not of interest. Examples of such enrichment media are MacConkey for enteric bacteria and thioglycollate broth for anaerobic organisms.

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By their very nature these tests are slow because the relevant organism must be isolated before it can be identified and checked for its antibiotic sensitivities. These steps may take as much as several days and this period can be critical in regard to bacterial diseases of humans

10   and animals.

Enumeration and identification of microorganisms as well as the determination of their susceptibility to antibiotics are the main goals of diagnostic medical microbiology. Numerous techniques, tests and media

15   have been developed in order to achieve these goals. However, none of the currently applied tests allow the fulfillment of these tasks by a short-term (i.e., minutes or hours) procedure. A total viable count of bacteria requires 18-24 hrs using present state of the art methods. The estimation of microbial biomass through the determination of adenosine

20 · triphosphate (ATP) content is not specific and does not allow the detection of less than 10^{4-5} bacteria per ml. Enumeration of a specific species of bacteria usually requires selective media and long periods of incubation. The final identification of the isolated colonies and the

25   determination of their antibiotic susceptibility often requires another cycle of growth and additional tests. Thus, the complete procedure for enumeration, identification and determination of antibiotic susceptibility of bacteria normally requires several days. In contrast, to the more lengthy procedures currently available, modern medicine has long sought

30   rapid diagnostic tools that would allow the rapid determination of the presence and identification of the bacteria causing an infection as well as information on their susceptibility to different antibiotics in a matter of minutes or hours.

35   A wide variety of alternative techniques have been investigated and developed in the continuing attempt to devise methods which overcome the

numerous drawbacks of the conventional culture approach. Light microscopy is used to detect bacteria in clinical specimens and can be used to differentiate between the major groups of bacteria. This technique however does not discriminate between dead and living bacteria and
5 the detection limit is usually above 10^7 cells/ml. Immunological methods have been successfully developed to detect specific species and genera which have surface antigens which are distinguishable by specific antibody binding, but such procedures require a relatively high concentration of bacteria and strain specific antibodies.
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Other tests have been developed based on detection of metabolic products of bacteria such as nitrites, nucleotides such as adenosine triphosphate, $^{14}CO_2$ released from ^{14}C-labelled substrates, and specific enzymes. The disadvantages of these techniques are many and include:
15 ATP can be degraded by enzymes in the test sample, ATP can originate from nonbacterial sources such as tissues of the host, radioactive materials present a biohazard, and enzymes with similar activity can arise from the host.

20   Particle counting instrumentation has also been applied to detection of bacteria. Such instrumentation measures pertubations in an electrical current across a small orifice caused by the presence of particles in a fluid flowing through the orifice. Besides requiring the use of a
25 complex and expensive apparatus, this method is highly nonspecific and requires a high level of care and particle-free conditions.

Other types of instrumentation measure the growth of bacteria in special liquid media. The instruments involved make periodic
30 turbidimetric measurements of increases in turbidity indicating the presence of growing bacteria. Here again the method is non-specific and requires a high concentration ($\geq 10^7$ cells/ml) of the bacteria in question.

35   In light of the foregoing summary of some demands and limitations of conventional microbiology, there is a continuing, long-felt need for a

rapid, sensitive, accurate and economical technique and means for the specific quantitative determination of living bacteria in the milieu in question. Despite the rapid advance of analytical techniques in closely related fields, as represented by the development of the

5   radioimmunoassay, spectrophotometry, fluorometry, microcalorimetry, and electrochemical techniques, the predominant technique used in bacteriological testing remains plate culture. This technique today still involves many of the same materials and methods used by Pasteur and other early microbiologists of the 20th century.

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Among biological systems, genetic recombination ordinarily does not occur between unrelated species of organisms. However, the new recombinant DNA technologies now permit the transfer of genes between related or unrelated organisms. The possibility of genetic alteration of

15   gene structure has particular application in the fields of industrial and medical microbiology. Diverse genetic information can now be gathered in vitro from various prokaryotic and eukaryotic sources in the form of DNA fragments and introduced into self-replicating genetic moieties known as cloning vectors. Alternatively, DNA fragments, or even intact genes, can

20   be constructed by synthetic chemical means to correspond to desired theoretical or known genetic sequences and then introduced into a selected vector. Bacterial plasmids or bacteriophages are commonly used cloning vectors. Plasmids are autonomous extra-chromosomal genetic units

25   consisting of circular strands of DNA which are found in most bacteria and some eukaryotes. Bacteriophages are DNA viruses which parasitize only bacteria. Hybrid genetic vectors can then be introduced into selected microbial hosts, which in turn serve as potential factories for the production of large amounts of the cloned DNA.

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Transformed microorganisms often do not express the foreign genetic information present in the cloning vector. Such non-expressing cloning vectors are desirable in those situations where expression of the foreign genome could produce a product deleterious to the host organism. A

35   non-expressing or low-expressing cloning vector represents a ready source of the foreign genetic material which can then be isolated and introduced

in turn into a suitable expression vector (i.e., a specially prepared or selected plasmid). By use of techniques known to the art, the foreign DNA is placed in a suitable location in an expression vector where the indigenous genetic sequence is such that the foreign genetic information

5   will be transcribed (i.e., mRNA produced from the foreign DNA) and translated (i.e., protein synthesis from the mRNA template) and the desired product coded in the foreign DNA obtained. Transformed microorganisms containing such expression vectors serve as factories for the manufacture of the foreign-DNA product.

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DNA can be cut _in_ _vitro_ at specific locations in preparation for insertion into an appropriate transfer vector by use of a class of enzymes known as restriction endonucleases. Restriction endonucleases are site-specific endonucleases which primarily cleave double-stranded

15   DNA, but in some instances cleave single-stranded DNA. For example, Class II restriction endonucleases cleave at specific sequences. In contrast, Class I restriction endonucleases appear to cleave DNA randomly and produce heterogeneous products. Various restriction endonucleases produce DNA fragments of different lengths and types. For example, some

20   restriction endonucleases cleave both DNA strands at the same point and produce the so-called "blunt end" DNA fragments. In contrast, other restriction endonucleases cleave one DNA strand several nucleotides away from the cleavage on the complimentary strand and produce "cohesive end" DNA fragments. Consequently, an accomplished practitioner of the

25   recombinant DNA art can, by creative selection of endonucleases for treatment of subject DNA, obtain desired DNA fragments which can then be joined together by the action of a DNA ligase. For ligation purposes, it may be desirable to add nucleotides to the ends of cut DNA fragments and

30   to add complementary deoxyribonucleotides to the ends of the cloning vector (i.e., in the process known as homopolymeric tailing). Another general method for obtaining a desired DNA fusion product is the addition of "adapter" or "linker fragments" to the ends of either or both the cloning vector or the DNA fragments to be cloned. Linker fragments are

35   small sections of DNA that contain one or more recognition sequences for restriction endonucleases. Thus, the cutting and splicing of DNA

containing genetic information is accomplished. The exact sequences of
foreign DNA which has been cloned and inserted into a host microorganism
can be determined by DNA sequencing procedures.

5   Foreign DNA material can be obtained for insertion into a transfer
vector by several ways. For example, DNA fragments directly obtained
from the parental source can be inserted into the appropriate vector.
Another method is to obtain mRNA from an active synthesis location in the
parent system and to then enzymatically synthesize (reverse
10   transcriptase) a single-stranded complementary DNA strand from the
isolated mRNA. A double-stranded DNA molecule is then synthesized from
the single-stranded template. Double-stranded DNA obtained in this
fashion is known as complementary DNA (cDNA). Once a desired DNA se-
quence is known, genes can also be chemically synthesized in vitro for
15   cloning/expression purposes in microbial, tissue, or cell culture sys-
tems.

      During recent years there has been dramatic progress in the area of
molecular biology due to the advent of recombinant DNA technology. Some
20   of these efforts have been directed towards diagnostic tests. Various
inherited disorders can be detected in human embryos, for example, and
even the carrier state can be uncovered in parents not showing the
disorder itself through the use of restriction endonucleases and nucleic
25   acid hybridization techniques. The ascertainment of the presence of a
given type of bacterium using DNA or RNA specific probes and nucleic acid
hybridization technology is also under development. However, the use of
novel genetic constructs, especially those made partly or wholly by
artificial means (in vitro) to detect the presence of specific organisms
30   has not been reported and is the basis of the present invention. A
preferred embodiment of the invention employs the luminescent system from
Virbio fischeri. The technique we have developed is by no means limited
to that system. Any exogenous genetically-introduced system showing
marked increase of expression in host   bacteria whose presence or
35   absence is to be determined can also be used.

The literature is replete with publications concerning bioluminescence and chemiluminescence (c.f., _Bioluminescence in Action_, P.J. Herring, ed., Academic Press, New York, NY 1978; _Bioluminescence and Chemiluminescence_, M.A. de Luca and W.D. McElroy, eds., Academic Press,

5   New York, NY, 1981). United States Patents Nos. 3,958,938; 3,470,373; 3,567,581; 3,959,081; 3,567,586; and 4,144,134 relate to phosphorescence and chemiluminescence.

The literature treating various aspects of genetic engineering, and

10   more particularly concerning recombinant DNA, is rapidly evolving. Various patents have issued in the area related to vectors, recombinant compositions, methodology, and novel microorganisms. Examples of such patents are: U.S. Patent Nos. 4,082,613; 4,184,917; 4,190,495; 4,195,125; 4,237,224; 4,468,464; 4,259,444; 4,262,090; 4,262,731;

15   4,273,874; 4,259,444; 4,262,090; 4,262,731; 4,273,874; 4,321,365; 4,399,216; 4,340,674; 4,506,013; 4,503,151; and 4,504,584. U.S. Patent No. 3,930,956 involves the transfer of raw bacterial DNA to an auxotroph. The detection of phage-induced lysis of bacteria is described in U.S. Patent No. 4,104,126.

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In 1983 Engebrecht _et_ _al._ (J. Engebrecht, K. Nealson and M. Silverman 1983, Bacterial Bioluminescence: Isolation and genetic analysis of functions from _Vibrio fischeri_, Cell _32_:773-781) described a variety

25   of recombinant plasmids constructed by ligating _Bam_Hl restriction fragments of _Vibrio fischeri_ (MJ-1) DNA with the plasmid pACYC184. These recombinant plasmids were introduced into _E. coli_ where they expressed and produced luminescence at a level comparable to that of _Vibrio fischeri_. Additional publications concerning bacterial luminescence

30   include: Belas, R. _et_ _al._ Sci. _218_:791-3 (1982); Evans, J.F., _et_ _al._ In vitro synthesis of subunits of bacterial luciferase in an _Escherichia coli_ system. J Bacteriol. _153_:543-545(1983); Engebrecht, J. and M. Silverman, Identification of genes and gene products necessary for bacterial bioluminescence. Proc. Natl. Acad. Sci. USA, _81_: 4154-4158

35   (1984); Engebrecht, J., _et_ _al._ Measuring gene expression with light. Sci. _227_:1345-47 (1985). Luminescent systems not involving luciferase are

known [DeSole, P., et al.,J. Clin. Lab. Automation 3: 391-400 (1983)].
In these publications there is no mention of making use of this technique
for the determination of bacteria; nor to the best of the inventors'
knowledge is the use of luminescing genetic elements for the determina-
5   tion of bacteria or other microorganisms suggested in any other publica-
tion.

It is the object of the present invention to provide a short-term
test that allows the specific identification of bacteria. It is a
10   further object of the invention to provide a highly sensitive test
capable of detecting and identifying bacteria in low concentration. It
is still a further object of the invention to provide such a test that
enables a quantitative estimation of the determined bacteria. It is yet
another object of the invention to provide a test for the determination
15   of the susceptibility of bacteria to antibiotics. These and further
objects are manifest in the following description and particularly
delineated in the appended claims.

GENERAL BACKGROUND OF THE INVENTION

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Various plants and animals exhibit biological chemiluminescence.
Bioluminescence is found in microorganisms [i.e., some bacteria (mostly
marine forms, e.g., Vibrio fischeri), fungi, and dinoflagellates],
25   insects (e.g., the firefly, Photinus pyradis), some crustaceans (i.e.,
Cypridine hilgendorfi), jellyfish, worms and other invertebrates and even
in mammals. Although the biochemical mechanism of luminescence is known
to vary (i.e., the luminescence system found in bacteria is different
from that found in fireflies and dinoflagellates), light production in
30   living organisms is most frequently catalyzed by the enzyme luciferase.
Bacterial luciferase is a mixed function oxidase, consisting of two
different subunits each with a molecular weight of approximately 40,000
daltons.

35   In the bacterium Vibrio fischeri, the synthesis of the enzymes
participating in the luminescence system is regulated by a small sensory

molecule, named autoinducer. During growth the autoinducer is accumulated in the growth medium. When the autoinducer reaches a critical concentration, induction of the luminescence system occurs, resulting in approximately 1000 fold increase in light production.

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The preferred element of the new test is a fragment of DNA carrying the luminescence system of a luminescent bacterium, usually a marine bacterium, e.g., Vibrio fischeri.

10   An extracellular DNA fragment carrying the luminescence genes does not, of course, luminesce but upon transferring it to a suitable living host by transduction or transformation, the host's genetic and synthetic machinery can utilize the DNA fragment thereby causing light to be emitted. Intracellular gene segments can also be used if in the test

15 organisms they are poorly expressed or unexpressed and only become expressed after some genetic transfer of exogenous DNA to the organism whose presence or absence is to be determined. This latter method employs conjugation, i.e., mating and genetic transfer from one cell to another.

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The introduction of DNA into a bacterial cell by bacteriophage infection (transduction), by transformation or by conjugation, is usually limited with respect to the donor source: DNA transfer occurs among a

25 group of strains of the same species or among closely related species. Some factors acting to limit such transfer include the presence of DNA restriction-modification systems in many bacterial species and/or their strains, the dependence on host factors for the replication of the introduced DNA and appropriate bacteriophage receptors in the bacterial

30 wall upon which bacteriophage can adsorb and thereby properly inject their genetic material into the host.

Conjugation and transformation are not particularly strain specific. Some plasmids can be transferred by conjugation not only to the same

35 species but also to related ones. There are even several plasmids which can be transferred to distant species by conjugation. Transformation is

potentially even broader than conjugation since a wide range of bacteria take up extracellular DNA. Many, if not most, that are at present not known to do so may in fact be transformable under specific conditions. For example, E. coli does not normally take up DNA efficiently. However,

5   after calcium shock this bacterium can be induced to do so at a high level of efficiency. A limiting factor in using transformation is the ability of the foreign DNA to express, replicate or integrate its genetic information into the host genome.

10   Unlike transformation and conjugation, transduction is quite strain or species specific. Some bacteriophages infect several species of bacteria which are usually close relatives; most infect only a particular subset of strains of a single species. By using different kinds of strains of bacteriophage which infect subsets, it is possible to arrange

15   the strains of a bacterial species into classification schemes. This is called phage typing.

Recombinant DNA technology and molecular genetics allow the introduction of genes whose products are easily assayable. Thus, the intro-

20   duction of a gene such as lac z of Escherichia coli into a bacterium can lead to expression of the introduced gene. If for example a bacteriophage is used to introduce the gene, then the course of the subsequent infection can be measured by analyzing the extent of

25   beta-galactosidase formation. Even though the bacterial cell itself may possess a gene for this enzyme, these measurements can be performed because the endogenous level can be depressed by appropriate media and the propagation of the bacteriophage genetic material leads to multiple copies of the gene.

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Although numerous exogenous genetic systems may be utilized in the invention, the luminescent system of Vibrio fischeri is particularly useful and illustrative of our method for ascertaining the presence or absence of a given bacterial species. Only a few bacterial species

35   contain genes allowing them to convert chemical energy to light. Most of these species are marine. The vast majority of bacterial species do not

contain such systems and are dark. If the genes for light production are introduced and express themselves in a species with no such capability, then emission of light will result. The background interference in such a system is extremely low (chemical luminescence). Since very low levels

5   of light can be detected and measured, tests based on this method should be sensitive and quantitative.

SUMMARY OF THE INVENTION

10   In accordance with the invention, there are provided methods and compositions useful for the determination of a specific type or group of bacteria in a milieu wherein exogenous DNA containing a luminescence system or other genetic system derived from a suitable donor organism is transferred by transformation, transduction, or conjugation (genetic

.15 means) to a host organism lacking or containing relatively low levels of endogenous DNA. The entry of the donor genetic material into the organism which it is desired to determine leads to the expression of the introduced system. The expression of such genes allows one to detect the presence and numbers of such organisms. In addition, if antibiotics are

20   added at the time of entry, the susceptibility of the host organism to the antibiotic can be determined. A preferred embodiment of the invention involves the transference of a luminescent system, or part thereof, obtained from a luminescent bacterium (or other source), and

25   used as a source for transferring this system into a non-luminescent host microorganism with subsequent expression of luciferase or other system in the host and concomitant production of light or other marker. The invention can also be used to detect antibiotics in a test sample, to assay microorganisms for antibiotic susceptibility, or to assay

30   compositions for antibiotic activity.

Alternatively, any other genetic system which is absent or poorly expressed until entry into the bacteria whose presence it is desired to ascertain may be used. For example, introduction of genes coding for

35   enzymes or proteins (e.g., beta-galactosidase of E. coli or the egg albumin protein from chickens) whose expression can be monitored by enzymatic assay, or by immunoassay can be used. These measurements may

be based on changes in pH or changes in ion concentration for species other than H^+; for the production of a specific molecule (e.g., amino acid, base, lipid, etc.) or the degradation and disappearance of a specific molecule; the production of gas (e.g., H_2 by E. coli); the
5   formation or disappearance of color (e.g., p-nitrophenol from p-nitrophenyl phosphate by alkaline phosphatase) or a molecule with particular light absorption properties at given wavelengths or radio- actively labelled molecules; the production or disappearance of secondary molecules dependent on primary products.

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     Luminescent bacteria are mainly marine, a typical example being Vibrio fischeri. The DNA containing a luminescence system derived from a luminescent bacterium, firefly, dinoflagellate, or luminescent fungi, etc. may be natural, in which case it is derived as such from the
15   luminescent organism. Alternatively, the DNA containing a luminescence system or part thereof derived from a luminescent bacterium or other source may be an artificial recombinant or synthesized DNA with the luminescence system or part thereof being derived from the luminescent source.

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     In cases involving a bacterial luminescence system, it may be desir- able to include in the medium an amount of autoinducer of the luminescent bacterium from which the luminescence system or part thereof is derived,
25   in order to avoid any prolonged incubation time which would otherwise be required for the formation of the autoinducer inside the host organism. If desired, it is also possible to add an aliphatic aldehyde to the milieu in order to accelerate or increase the expression of the luminescence system or part thereof derived from a luminescent bacterium.
30   In those instances where a foreign genetic marker system other than a luminescence system is introduced into a suitable host, it may be advantageous to introduce intermediates, precursors, enzymatic substrates, or other ingredients to facilitate or enhance expression of the marker genetic system or detection of the expression.

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     In the host organism the DNA containing a luminescence system or part thereof derived from a luminescent bacterium or other source is

transcribed by RNA polymerase to form mRNA that can then be translated into the luminescence system proteins. The luminescence level of even a single bacterium can be detected with, for example, the aid of a scintillation counter.

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It is thus seen that, in accordance with the invention, luminescence is produced upon interaction between two non-luminescent components, namely the organism to be determined and said DNA containing a luminescence system or parts thereof derived from a luminescent bacterium

10 or other source. Consequently, any luminescence that exceeds the weak background chemiluminescence is conclusive of the presence of the host organism. Conversely, the absence of any luminescence beyond the background level of chemiluminescence is conclusive of the absence of the host organism. Such a technique has never been suggested before.

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It is possible, in accordance with the invention, to construct standard curves showing, for example, the correlation between the level of luminescence or other expressed system and the concentration of the bacteria in question. Using such curves the amount of bacteria in the

20 milieu can be closely approximated.

The transfer of the DNA containing a luminescence or other marker system or parts thereof derived from a suitable donor to the host orga-

25 nism may be mediated by transformation, transduction or conjugation. A large number of microbial genera serve as hosts for bacteriophages and retain or accept plasmids. Examples of such genera are: Escherichia, Aerobacter, Salmonella, Shigella, Klebsiella, Proteus, Pseudomonas, Staphylococcus, Streptococcus, Chlamydia, Mycoplasma, Pneumococcus,

30 Neisseria, Clostridium, Bacillus, Corynebacterium, Mycobacterium, Camphybacter, Vibrio, Serratia, Enterobacter, Providencia, Chromobacterium, Brucella, Yersinia, Haemophilus, and Bordetella.

For transduction bacteriophages are constructed that are specific to

35 the bacteria to be determined. If desired, a series of tests may be run

each with a different bacteriophage, either sequentially or simultaneous-
ly.

Some bacteriophages may be constructed by packaging DNA containing a
luminescence system or parts thereof or other donor marker system derived
from a luminescent bacterium or other suitable source in the proper
bacteriophage capsid. Alternatively, the bacteriophage may be construct-
ed by recombinant DNA technology using restriction endonuclease with or
without subsequent packaging. Alternatively, the bacteriophages contain-
ing all or part of the luminescence or other system may be constructed
through naturally occurring genetic recombinational mechanism. This can
also involve the use of both artificial recombination and natural recom-
bination.

For conjugation the invention provides bacterial strains containing
a luminescence system or parts thereof in a transferable replicon. The
replicon may be the bacterial chromosome or a plasmid. The luminescence
system or parts thereof may be introduced by a bacteriophage or by
genetic recombination, either natural or artificial, such that the
luminescence genes are covalently linked to the transferred genetic
material. Alternatively, any other genetic system whose primary or
secondary products can be measured as described earlier can be used in a
like fashion. For the sake of illustration, luminescence is given as a
relevant and particular example of the more general principle underlying
this invention.

For example, it is possible to use for conjugation in accordance
with the invention, bacterial strains that are Hfr's (high frequency of
conjugation), and which are lysogenic for a temperate bacteriophage
carrying the luminescence system. Obviously other Hfr's or donors can be
made through genetic recombination, either natural or artificial, or
through the addition of genetic material such that the Hfr or donor
carries the luminescence system or parts thereof.

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In the performance of a test according to the invention herein described, the host organisms perform metabolic activities that are involved in the formation of luminescence or other donor systems; this includes protein synthesis and a process that generates a reducing power. Antimicrobial agents that affect these processes or alter cell integrity abolish or reduce the formation of the donor genetic system (i.e., luminescence, etc.). Consequently it is possible in accordance with the invention to determine the susceptibility of bacteria to various antibacterial agents, e.g., antibiotics. To this end the specific type or group of bacteria to be tested are subjected to DNA transfer as specified in the presence of a given antibacterial agent, and the kinetics of the expressed donor genetic system (i.e., luminescence), which are a function of the protein synthesis capacity and the general metabolism of the host bacteria are then determined. Preferably simultaneous tests are run, one in the absence of any antibacterial agent and several others each in the presence of a particular antibacterial agent. By comparing the results of such tests conclusions can be drawn on the susceptibility of the tested bacteria to any of the tested antibacterial agents.

DESCRIPTION OF SPECIFIC EMBODIMENTS

1. Materials and Methods

I. Growth media

    A.   LB (Lennox broth). (Lennox, E.S. 1955, Transduction of linked genetic characters of the host by bacteriophage. Pl. Virology 1:190-206). [10 g tryptone; 5 g NaCl; 5 g yeast extract; 1 l distilled H_2O; 4 ml 1N NaOH]

       1.   For petri dishes: 15. g/l agar is added.

       2.   Autoclaved at 121°C for 15 minutes.

       3.   As additions where indicated: (a) ampicillin 30 mg/l; (b) chloramphenicol 30 mg/l; (c) tetracycline 15 mg/l.

B.   FT [10 g tryptone; 5 g NaCl; 2 g maltose; 10 ml 1M $MgSO_4$; 1 l distilled H_2O]

    1.   Autoclaved at 121° C for 15 minutes.

    2.   As additions where indicated: (a) ampicillin 30 mg/l; (b) chloramphenicol 30 mg/l; (c) tetracycline 15 mg/l.

C.   T for bacteriophage [10 g tryptone; 5 g NaCl; 10 g agar; 1 l H_2O]

    1.   Autoclaved 121° C for 15 minutes.

D.   TA (Top Agar) [10 g tryptone; 5 g NaCl; 6.5 g agar; 1 l H_2O]

    1.   Autoclaved 121° C for 15 minutes.

E.   Complex liquid medium (ASWRP) and the complex solid medium are described by Ulitzur et al. (Ulitzur, S., Weiser, I. and S. Yannai 1980: A new, sensitive and simple bioluminescence test for mutagenic compounds. Mutation Res. 74:113-124).

II.   Strains and DNA

A.   Bacterial strains

    1.   MM294 E. coli K12 endA thi pro hsdR (Backman, K., Ptashne, M. and W. Gilbert, 1976: Construction of plasmids carrying the cI gene of bacteriophage. Proc. Natl. Acad. Sci. USA 73:4174-4178.

    2.   JM101 E. coli K12 endA thi sbcB supE _____ lac-pro/F'traD proAB lacI lac z ΔM15 (Vieira, J. and J. Messing, 1982: The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268).

3. W3110 E. coli K12 F⁻ wild type (Yanofsky, C., Horn, V.,
    Bonner, M. and S. Stasiowski, 1971: Polarity and enzyme
    functions in mutants of the first three genes of the
    tryptophan operon of Escherichia coli. Genetics
    69:409-433).

4. AT2448 E. coli K12 HfrH thi met.

5. CSHI Escherichia coli K12 lac trp strA (Miller, J. H.
    1972: Experiments in Molecular Genetics. Cold Spring
    Harbor Laboratory, Cold Spring Harbor, N.Y.)

6. Proteus vulgaris.

7. Staphylococcus albus.

8. Aerobacter aerogenes 62-1 (Gibson, F., 1968: Chorismic
    acid, P. 94-97. In W.E.M. Lands, (ed.), Biochemical
    preparations, vol. 12. John Wiley and Sons, Inc., New
    York).

B. Bacteriophage strains

1. λ cI⁺ wild type.

2. λ Charon 30 (Maniatis, T., Fritsch, E.F. and J. Sambrook,
    1982: Molecular Cloning. Cold Spring Harbor Laboratory,
    Cold Spring Harbor, N.Y.).

3. λ cI857 S7 (Miller, J.H., 1972: Experiments in Molecular
    Genetics. Cold Spring Harbor Laboratory, Cold Spring
    Harbor, N.Y.).

4. λ cI⁻ b2.

5.   λ cI857 S7 plac5 (Miller, op. cit.)

C.   Plasmids and DNA

5   1.   pBR322 4.36 Kb, containing genes for resistance to
          ampicillin and tetracycline (Bolivar, F., Rodriguez, R.L.,
          Greene, P.J., Betlach, M.C., Heynecker, H.L., Boyer, H.W.,
          Crosa, J.H., and Falkow, S., 1977: Construction and
          characterization of new cloning vehicles. II. A multipur-
10   pose cloning system. Gene 2:95-113). After transforma-
          tion, these resistances are convenient for detecting the
          presence of the plasmid or recombinants of them.

     2.   pACYC184 A plasmid of 4.3 kb containing genes for resis-
15   tance to tetracycline and chloramphenicol (Chang, A.C.Y.,
          and Cohen, S.N., 1978: Construction and characterization
          of amplifiable multicopy DNA cloning vehicles derived from
          the   P15A   cryptic   miniplasmid,   J.   Bacteriol.
          134:1141-1156).

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III. Bacteriophage techniques

     In the main the techniques described by Miller (loc. cit.) were used
25   except that the medium for plates was T and the liquid medium was
     FT.

IV. Preparation of bacterial DNA

30   The method of Marmur was used (Marmur, J. 1961: A procedure for the
     isolation of deoxyribonucleic acid from microorganism. J. Molec.
     Biol. 3:208-218).

V.   Use and source of restriction endonucleases
35

Restriction endonucleases were purchased from BRL, New England
Biolabs, Boehringer and Amersham. Digestion of DNA by such enzymes
was according to the suggestion of New England Biolabs catalogue
(1982) except that 100 μ g/ml gelatin replaced bovine serum albumin
5 in all cases. DNA concentration did not exceed 2 μ g/20 ul.

A.   Agarose gels

1.   2 g agarose (Sigma catalogue No. A6013).

10

2.   200 ml TAE buffer (1X); TAE buffer 10X: (a) 48.4 g Sigma
      7-9 Tris (hydroxymethyl aminomethane) catalogue No. T1378
      (Tris HCl); (b) 27.2 g sodium acetate; (c) 10.5 g NaCl;
      (d) 7.5 g disodium ethyelene diaminetetraacetic acid; (e)
15   17 ml concentrated HCl; (f) 925 ml H_2O distilled; (g) pH
      8.2 at $25^{o}C$;

      (i)   To each sample of DNA (1-20 1) was added 4 μl of
            bromophenol blue in glycerol.

20

      (ii) The gels were run under buffer at 40-250 mA until the
            dye had run an appropriate distance.

25   3.   Where necessary, λ cI857 S7 DNA was cut with restriction
            endonuclease HindIII or EcoRI and used as a molecular size
            standard. Regression analysis was used to establish the
            relation between the log of the molecular length and
            migration distance. The standard was considered accept-
30   able when the correlation coefficient was greater than
            r=0.99.

      4. . The gels were stained by placing them in plastic trays
            with sufficient water to cover them. Seven drops of
35   ethidium bromide (Sigma E8751) at a concentration of 10
            mg/ml were added and dispersed by gentle tilting of the

20

pan. After 15 minutes the excess dye was removed by
suction, the gel rinsed with water and covered again with
water without dye. After 15 minutes the water was removed
and the DNA visualized by placing the gel on a long wave
5   UV light box. Photographs were taken with Kodak Tri X pan
Professional Film.

VI. Ligation

10   DNA's digested by restriction endonucleases were precipitated by the
addition of one-tenth volume of 3N sodium acetate and 2.2 volumes of
distilled 96% ethanol. After freezing at -70°C in a dry ice-ethanol
bath, the Eppendorf tubes were centrifuged for 10 minutes in an
Eppendorf centrifuge, the supernate carefully removed and discarded,
15   the pellet washed with 100 μl 70% ethanol, centrifuged for 3 min-
utes, the supernate removed and discarded, and the pellet dried for
10 minutes in a dessicator under vacuum. The dry pellets were
resuspended in water.

20 .   Ligations were performed in 20 μl containing 4 μl of a five-fold
concentrated kinase-linker buffer (Maniatis et al., loc. cit.) and 1
μl of DNA ligase (New England Biolabs., Catalogue No. 202; 400
units/ml) at 25° C for 12-16 hours. The DNA concentration was
25   usually 0.5 - 1.0 μg/20 μl reaction.

VII. Preparation of plasmid DNA

A.   Preparation of crude plasmid DNA from small volumes
30

1.   The bacterial strain containing a plasmid is grown over-
night at 37°C in LB· medium containing an antibiotic to
which the plasmid confers resistance so that only bacteria
with the particular plasmid reproduce.

35

2.　1 ml of the cell suspension is centrifuged for 20 seconds in an Eppendorf centrifuge.

3.　The supernate is discarded and the cell pellet resuspended in 50 μ1 of STET buffer (8% sucrose, 5% Triton-100, 50 mM disodium ethylenediamine tetraacetic acid, 50 mM Tris (hydroxymethyl aminomethane)-HCl, pH 8.0).

4.　4 μ1 of 10 mg/ml lysozyme (Sigma, catalogue No. L6876) is added.

5.　The suspension is immersed in boiling water for 40 seconds.

6.　The suspension is immediately centrifuged in an Eppendorf Centrifuge, model 5412, for 20 minutes.

7.　The supernate is carefully removed to a fresh tube, the pellet is discarded.

8.　50 μ1 of isopropanol are added and the contents mixed. The tube is placed at -70°C for 10 minutes in an ethanol dry-ice bath.

9.　The tube is centrifuged for 10 minutes in the Eppendorf centrifuge.

10.　The supernate is removed and discarded. The pellet is dried under vacuum for 5 minutes.

11.　The pellet is resuspended with 50 μ1 of 0.3 M sodium acetate.

12. 150 μl of distilled ethanol are added, the contents mixed and the tube placed at -70°C in the ethanol dry-ice bath for 10 minutes.

13. The tubes are centrifuged for 10 minutes, the supernate carefully removed and discarded.

14. The pellet is rinsed by adding 200 μl of cold (-20°C) 70% ethanol without disturbing the pellet.

15. The tube is centrifuged for 2 minutes, the supernate removed and discarded, and the pellet dried under vacuum for 10 minutes.

16. The pellet is resuspended with 20 μl of H_2O.

17. For most plasmids about 1 μg of fairly pure plasmid DNA is recovered.

B. Preparation of highly purified plasmid DNA by cesium chloride-ethidium bromide equilibrium density-gradient centrifugation.

1. Grow a 1 liter culture for 12-16 hrs in LB medium with an appropriate antibiotic so that only those bacteria breed which contain the particular plasmid.

2. Chill and centrifuge at 5000 xg for 10 minutes at 0°C in four 250 ml centrifuge bottles. Discard the supernate.

3. Wash the pellet with 20 ml of Tris-HCl buffer pH 7.9, 25 mM containing 1 mM ethylenediaminetetraacetic acid (EDTA).

4. Resuspend each pellet in 7.5 ml of 25% sucrose made up in
   50 mM Tris-HCl pH 8.0 and transfer each resuspended pellet
   to a 40 ml polypropylene tube (Oak Ridge type).

5. Add 1.5 ml lysozyme (Sigma catalogue No. L6876). The
   lysozyme is made fresh at 5 mg/ml in 0.25 M Tris-HCl pH
   8.0. Mix and hold for 5 minutes at 0°C.

6. Add 3 ml of 0.25 M Na EDTA pH 8.0. Mix and hold for 5
   minutes at 0°C.

7. Warm to room temperature and add 1 ml of sodium lauryl
   sulfate (SDS) solution (12.5% in 50 mM Tris-HCl, pH 8.0).
   Mix gently by inversion and hold for 15 minutes.

8. Chill to 0°C and add 2.5 ml of 5 M NaCl. Mix gently by
   inversion and hold for 30 minutes at 0°C.

9. Centrifuge at 43,000 g (19,000 rpm in a Sorvall SS-34
   rotor) for 45 minutes at -4°C.

10. Decant the four supernates ($\sim$12 ml each) into four 40 ml
    polypropylene tubes. Discard the pellet. Dilute with an
    equal volume of water.

11. Add to each tube 50 μl of RNAase A, 10 mg/ml. (Sigma
    catalogue No. R5503) after inactivation of any DNAase
    activity through heating in T1 buffer at 80°C for 20
    minutes. T1 buffer is: 10mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.1%
    gelatin, 6 mM Tris-HCl, pH 8.0. Hold at 37°C for 1 hour.

12. Chill and add 4 ml of phenol that has been saturated with
    STE buffer. STE buffer is 10 mM Tris-HCl pH 7.9, 10 mM
    NaCl, 1 mM EDTA. Mix well and centrifuge at 16,000 g for
    15 minutes at 0°C.

13. Carefully remove the upper layer (aqueous phase) from each tube into a single 250 ml centrifuge bottle. Add a one-quarter volume of 5 mM NaCl and mix. Add an amount of ethanol that is twice the total volume of the mix. Mix. Hold at -20°C for at least 12 hours.

14. Centrifuge at 9000 rpm for 45 minutes. Remove and discard the supernate. Add 50 ml of 70% ethanol and gently wash the pellet. Spin 20 minutes at 9000 rpm. Remove and discard the supernate. Drain the pellet and then dry it under vacuum for 15 minutes. Resuspend the pellet in 5 ml of STE buffer.

15. Make the DNA containing solution to 46.2 ml with STE. Add 45 g of CsCl. Add 1.8 ml of ethidium bromide (Sigma, catalogue No. E8751) at 10 mg/ml in STE buffer. Adjust the solution so that its refractive index r_D is between 1.3870-1.3880 (ρ =1.565-1.576).

16. Pipet 10 ml of the mix into each of six 3 x 5/8 inch polyallomer tubes. Cap tubes, fill with paraffin oil and centrifuge in a Ti 50 rotor at 38,000 rpm for 48 hours at 15°C.

17. Remove the lower band (viewed with an UV lamp) using a 1 inch 20 gauge needle and a 3 ml syringe. Extract the ethidium bromide 4 times with an equal volume of isopropanol (stock kept over CsCl-saturated STE buffer).

18. Dilute the separate aqueous solution with 4 volumes of water and add twice the total volume of ethanol (96%). Hold for 12 hours at -20°C. Centrifuge in 40 ml polypropylene tubes at 19,000 rpm for 1 hour at -4°C. Decant and discard the supernate. Drain the tube and then

dry under vacuum for 15 minutes. Resuspend the DNA in 1
ml of STE buffer.

VIII. Transformation (M. Mandel and A. Higa, 1970: Calcium dependent
bacteriophage DNA infection, J. Molec. Biol. 53:159-162).

The bacterial strain to be transformed is grown overnight at 37°C in
LB medium. It is diluted 1:200 into fresh LB and grown with
aeration at 37°C until its absorbance, A, at 600 nM is 0.5. The
culture (original volume) is chilled on ice, then centrifuged in a
Beckman centrifuge at 7000 rpm for 7 minutes at 0°C in a JA 20
rotor. The cells are resuspended in the same volume of ice cold 0.1
M $MgCl_2$, and centrifuged as before. The cell pellet is resuspended
in one-half of the original volume of 0.1 M $CaCl_2$ and kept on ice
for 30 minutes. The suspension is centrifuged as before and resus-
pended in one-tenth of the original volume of 0.1 M $CaCl_2$. These
cells are competent for transformation.

A 0.2 ml portion of these concentrated $CaCl_2$ treated cells is mixed
with 1-100 µl of solution containing 0.1-2 µg of DNA. The mixture
is held at 0°C for 30 minutes, heated to 42°C for 3 minutes in a
water bath and again placed on ice for an additional 20 minutes.
Then 0.8 ml of LB medium is added and the culture placed at 37°C for
1 hour to allow the expression of antibiotic resistance essentially
without cell division since the cells are recovering from the
calcium shock. Portions of the culture are placed on LB plates
(containing the appropriate antibiotics). Controls consist of
untransformed but Ca^{++} treated cells and cells transformed with a
known amount of an uncleaved plasmid.

IX. Construction of pBTK5 and transformation into MM 294 E. coli.

DNA (8 µg) from Vibrio fischeri strain MJ1 was cleaved with 30 units
of restriction endonuclease SalI for 3 hrs at 37°C. DNA (6 µg) of
plasmid pBR322 was similarly digested. Samples were tested by

agarose gel electrophoresis to check that the digestions had gone to
near completion. The cleaved DNA's were precipitated with sodium
acetate-ethanol and the dry pellets resuspended in H_2O. A 2 µg
amount of V. fischeri DNA was ligated to 0.5 g of pBR322 DNA using

5   400 units of DNA ligase. The reaction was terminated by adding 200
µl H_2O and 40 µl STE saturated-phenol. The mixture was vortexed
and centrifuged. The supernate was precipitated with sodium
acetate-ethanol and the pellet of ligated DNA was resuspended in 40
µl of H_2O.

10

Strain MM294 was grown and prepared for transformation. The control
transformation contained 1 µg pBR322 DNA (loc. cit.). After trans-
formation the cells were plated on LB plates containing ampicillin.
Cells without added DNA gave no colonies. 2.64 x 10^6 transformants/

15   µg pBR322 DNA were obtained in the control transformation. The
ligated mixture gave 3.2 x 10^4 transformant colonies/ µ g bacterial
DNA and 6 x 10^4 total transformants were obtained. By checking for
the insertional inactivation of the tetracycline resistance gene
(inserts of foreign DNA inactivate the gene since the SalI site is

20 .   in this gene) it was found that about 5% of these transformants
contain inserts of V. fischeri DNA. Thus about 3000 inserts were
obtained. Seven of the colonies were luminescent.

25   After anaylsis of the DNA contained by these 7 colonies, one strain
containing a plasmid (pBTK5) that has a single insert of approxi-
mately 8 Kb and two SalI sites was chosen. These results are in
line with those previously described by Engebrecht et al. (loc.
cit.).

30

X.   Construction of pAChv-1 and transformation into MM294 E. coli.

CsCl-ethidium bromide purified plasmid pBTK5 DNA from MM294 strain
containing this plasmid obtained according to IX above, was digested

35   with restriction endonuclease SalI and a portion was checked by
agarose gel electrophoresis. pACYC184 plasmid DNA, similarly

prepared, was cleaved by SalI and analyzed as above. After ligation
and transformation into MM294 E. coli cells, colonies that were
resistant to chloramphenicol (from pACYC184) and that gave light
(30°C) were analyzed by standard methods. A strain containing a
5   plasmid (pAChv-1) that has the backbone of pACYC184 and the 8 Kb
light producing segment inserted in the SalI site of pACYC184 was
kept, and placed in permanent deposit (ATCC 53133).

XI.   Construction of λL1, λL4, λL5, λL28, λL32, λL35 and λL40.

10

Bacteriophage λCharon 30 DNA (0.3 μg), purified as described by
Maniatis (loc. cit.), was cleaved with restriction endonuclease SalI
and ligated to pBTK5 DNA (0.5 μg) obtained according to IX above,
similarly cleaved. After ligation the DNA was precipitated by
15   sodium acetate–ethanol, the pellet dried and resuspended in 5 μl
H_2O.

Packaging mix for DNA was prepared by the method of B. Hohn as given
in Maniatis (loc. cit.). The packaging of the ligated DNA was
20   carried out according to the protocol of Maniatis (loc. cit.) and
plated with MM294 grown to stationary phase in FT medium. Plaques
were checked for light production by transferring them to scintil-
lation vials containing 1 ml FT medium, 0.1 ml MM294 grown on FT
25   medium. Individual plaques had been transferred through the use of
a Pasteur pipette to 1 ml of 10 mM $MgSO_4$. A 0.1 ml of this was
transferred to a vial.

Phage strains giving light were plaque purified twice, plate lysates
30   were made and the phage preparations stored over several drops of
chloroform. These phages were then characterized for light
production during infection of·strain MM294 growing in FT medium at
27°C. Autoinducer was added to a duplicate vial (Nealson, K.H.,
Platt, T., and Hastings, J.W., 1970, Cellular control of the
35   synthesis and activity of the bacterial luminescent system. J.
Bacteriol. 104:313–322). λL1, λL35 and λL40 produce light at

times considerably later than the others, the light produced is relatively little and the amount of light emitted is greatly increased if the bacteriophage infection takes place in the presence of autoinducer. Phage strains λ L4, λL5, λ L28 and λL32

5 produce light within 15-20 minutes after infection, and the amount of light emitted increases with time and reaches a maximum after about one hour. There is no significant effect of autoinducer on the amount of light produced by these phages.

10 Since the fragment containing the light genes had been cloned from a digest with restriction endonuclease SalI, insertion of this fragment into λ Charon 30 digested with the same enzyme may occur in two different orientations. In one orientation the luciferase and aldehyde genes (Engebrecht op. cit.) are closer to the N gene of λ ,

15 while the regulatory genes of the lux operon are closer to the J gene of λ. In this orientation, the transcription of the aldehyde and luciferase genes will depend on transcription from the lux promoter(s) only, since the direction of transcription will be opposite to that of the P_L promoter of λ . In this case,

20 autoinducer should and does stimulate light production. λ L1, λ L35 and λ L40 are most likely phages with inserts in this orientation, since their light emmission is greatly stimulated by autoinducer.

25 The second possible orientation has the regulatory gene of the lux operon nearer the N gene of λ and the aldehyde and luciferase genes to the J gene side. Since the direction of transcription of the aldehyde and luciferase genes is the same as that of the P_L promoter of λ , these genes should be transcribed not only from their own

30 promoter but even more so from P_L. Thus, the addition of autoinducer to cultures of bacteria infected by these kind of phages should and does have little or no effect on light production.

Two of these phages, λ L4 and λ L28, have been characterized and

35 the orientation of their insert found to be that which was expected on the basis of the above physiological observations and theoretical

considerations. Phage DNA from λ L4 and λ L28 was prepared as described by Maniatis (op. cit.) using CsCl gradient purified phage as the source of DNA.

5   Digestion of these DNAs by restriction endonuclease SalI revealed three large fragments after electrophoresis in agarose gel. The two larger bands are the arms of λ Charon 30 that are necessary for phage propagation. The smallest band of about 8.8 kilobases is identical to a band from pBTK5 cut with the same enzyme. Thus the

10   inserted DNA piece in both phages seems identical to the piece that was originally cloned from Vibrio fischeri. The orientation of the insert in λ L4 and λ L28 was shown by restriction endonuclease cleavage using PstI. λ Charon 30 has the normal λ sequence until approximately base pair 23616 (Hendrix R.W., Roberts, J.W., Stahl,

15   F.W., and Weisberg, R.A., 1983: Lambda II, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Appendix II, pp 519-676) and then the deletion b1007 which extends from approximately base pair 23616 to 28312. From base 28312 until base 34449 the sequence is again normal. At base 34449 an earlier sequence repeats itself

20   and this repeat starts with base pair 22346. The repeat continues until base pair 23616 and then is deleted (b1007) until base pair 28312. Starting with this base pair the normal sequence continues until approximately base pair 35384 at which point a second, differ-

25   ent type of deletion, KH54, commences and removes DNA until base pair 37925. Then the normal sequence continues until approximately base pair 40502 at which point a third kind of deletion (nin5) removes DNA until base pair 43307. The sequence after this base pair is normal until the DNA end at base pair 48502 (these numbers

30   are given as the normal base pair coordinates of normal bacteriophage λ ; see Fig. 9). Thus λ Charon 30 contains 1 duplication and 4 deletions, two of which are identical. The coordinates for various restriction endonuclease cleavage sites (see Fig. 10) in λ Charon 30 are given in Hendrix (op. cit.) Appendix

35   III. They estimate the phage λ Charon 30 to be 46757 base pairs.

From published data the phage would seem to be 46331 base pairs. For our purpose this discrepancy is not important.

The pattern of restriction enzyme fragments obtained from PstI cleavage in λ Charon 30 can be calculated from the description of its DNA provided above. After removal of the internal SalI fragment and insertion of the lux operon SalI fragment this pattern is, of course, altered. The PstI sites until the base pair 22425 will be identical to normal λ and also λ Charon 30. There will be a fragment in λ Charon 30 of 4888 base pairs generated by the segment between the site at 22425 and 32009 (contains deletion b1007 which removes 4696 base pairs). This fragment will also occur in the "luminescent" phage described here. However, the DNA insert will alter the pattern between the PstI sites at base pair 22425 and the end (normal λ coordinates). After removal of the internal SalI fragment from λ Charon 30, no PstI sites will exist in the λDNA from base pair 32256 (the most distal PstI site remaining) and the end of the phage since the PstI site at base pair 37005 is deleted in λ Charon 30 by the KH54 deletion. There is one PstI site in the SalI fragment containing the lux operon and it is situated very near one end (about 200 bp from the end) and in the regulatory region of the operon.

Thus if the PstI site of the lux operon is near the last PstI site in λ , PstI cleavage will generate a small fragment of about 700 base pairs and a large fragment of about 18 - 19 kilo bases. This would be the expected cleavage pattern for λ L1, λ L35 and λ L40. Conversely, if the PstI site of the lux fragment is toward the distal end of the λ , then two fragments of about equal size (approximately 9 and 11 kilobase) should result. Cleavage of λ L4 and λ L28 DNA gave the latter pattern which confirms our hypothesis that the luciferase and aldehyde genes of the lux operon are located towards the proximal end of λ and have the same transcription orientation as transcription from the P_L promoter.

A final question that can be raised about the structure of λ L4 and λ L28 has to do with their SalI junctions between phage and insert DNA. λ Charon 30 has four SalI sites. These four are divided into 2 pairs. Within each pair of sites, the sites are separated by only

5   499 base pairs while the distance between pairs is close to 7000 base pairs. When λ Charon 30 was used as a vector and cleaved with SalI restriction endonuclease to generate λ L4 and λ L28, the fragment between the pairs will be replaced by the lux operon fragment. What is less clear is whether the recombinant phages

10   contain 2, 3, or 4 SalI sites. If there are only 2, then the junction between the lux operon and λ DNA was at the most proximal and most distal SalI sites of λ. If there are 4 SalI sites then the lux fragment is joined to the two innermost SalI sites of λCharon 30. Three SalI sites indicate that a pair of SalI sites

15   still exists at one end of the insert and a single SalI site at the other.

The 7Kb (kilobase) internal fragment of λ Charon 30 must always be

20   removed because, if it were not, the addition of the lux operon would lead to a phage DNA molecule that is too long to be packaged.

The small (499 base pair) fragment(s) produced when there remain 3

25   or 4 SalI sites, can be seen on a gel, but one cannot determine whether there are 3 or 4 sites since each pair gives an identically sized fragment. A solution to this problem is afforded by cleavage by restriction endonuclease ScaI, since ScaI cleaves λ DNA between the two SalI sites of a pair. Thus, if one or both of the ScaI

30   sites disappears, there is no or one pair left in the recombinant λ . There is no ScaI site in the lux operon. ScaI cleaves wild type λ at sites 16420, 18683, 25684, 27262 and 32801 base pairs. In λ Charon 30 the sites at 16420 and 18683 exist while the sites at 25684 and 27262 have been deleted. The site at

35   32801 occurs twice in λ Charon 30 since there are 2 pairs of SalI sites rather than the single pair in λ wild type. In λ Charon 30

there is a _ScaI_ site at base pair 28391 and at base pair 35819, besides those at 16420 and 18683. λ Charon 30 gives fragments of 16420, 2263, 9708, 7428 and 10938 base pairs after _ScaI_ digestion. A recombinant phage having the lux operon and only 2 _SalI_ sites remaining, should give fragments of 16420, 2263 and one of about 28000 base pairs after _ScaI_ digestion. The presence of 3 _SalI_ sites will give either a pattern of 16420, 2263, 9708 and approximately 19000 base pairs, or 16420, 2263, approximately 18500 and 10938 base pairs after _ScaI_ digestion, depending on whether the pair of _SalI_ sites still remaining is proximal or distal respectively. The presence of both pairs of _SalI_ sites will yield fragments of 16420, 2263, 9708, approximately 9300 and 10938 base pairs after _ScaI_ cleavage.

The results of analysis by _ScaI_ endonuclease shows that λ L4 contains 4 _SalI_ sites while λ L28 has 3. λ L28 retains the proximal pair of _SalI_ sites and the most distal _SalI_ site from λ Charon 30. This structure of λ wild type, λ Charon 30, λ L4 and λ L28 are shown diagramatically in Figures 9, 10, 11 and 12, respectively.

XII. Construction of an Hfr able to transfer luminescence.

Escherichia _coli_ K12 strain AT2446 which is an Hfr was made lysogenic for the luminescent bacteriophage λ L28. This was accomplished by simultaneously infecting AT2446 with λ L28 and λ cI^{+}. The latter phage is necessary to provide integration functions, the λ att site for integration, and an active repressor gene to allow lysogenization: all three of these are lacking in λ L28. Non-lysogenic cells were eliminated by subsequently challenging the cells with λ cIb2 which cannot itself lysogenize and which kills non-lysogens. Survivors that give off light, produce phages after induction by UV light, and carry luminescent genes were isolated.

XIII. Bioluminescence determination.

Luminescence _in vivo_ of aliquots placed in scintillation vials was measured by a photomultiplier photometer similar to that described by Mitchell & Hastings (Mitchell, G.W., and Hastings, J. W., 1971: A Stable inexpensive solid state, photomultiplier photometer. Anal. Biochem. $\underline{39}$: 243-250). When the luminescence intensity was below 10^6 quanta/sec, a scintillation counter (Packard Model 2001), operating without coincidence at the ^3H setting at 25°C, was used. The luminescence was expressed in quanta per second by using the Hastings and Weber standard (Hastings, J.W., and G. Weber, 1963: Total quantum flux of isotropic sources. J. Opt. Amer. $\underline{53}$:1410-1415).

XIV. Autoinducer preparation from $\underline{V}$. fischeri MJ-1.

To prepare cell-free conditional medium containing autoinducer, $\underline{V}$. fischeri MJ-1 cells were grown in ASWRP liquid medium with shaking at 22°C. Late logarithmic phase cultures (80-100 Klett units, Filter 66) that are highly luminescent were centrifuged at 10,000 g at 4°C for 15 minutes. The supernatant fluid was sterilized by passage through a 0.22 µm pore size membrane filter (Millipore Corp. Bedford, Mass.). These preparations were stored at 4°C for up to 30 days without loss of activity.

The bioassay for the $\underline{V}$. fischeri autoinducer was performed using the methods previously described by Nealson (Nealson, K.H., Platt, T., and Hastings, J.W., 1970: Cellular control of the synthesis and activity of the bacterial luminescent system. J. Bacteriol. $\underline{104}$: 313-322).

XV. Assay of beta-galactosidase.

The assay was performed according to Miller (op. cit.) using chloroform and SDS (sodium lauryl sulfate) to permeabilize the cells to o-nitrophenyl galactoside (ONPG).

34

XVI. Deposition.

Where indicated, constructions (i.e., plasmid and bacteriophage) of the invention have been placed on permanent deposit in the American
5   Type Culture Collection (ATCC), Rockville, MD 20852, U.S.A.

2. List of Figures

The invention is illustrated in the annexed graphical representa-
10   tions, which show:

Fig. 9 - λwild type Map of relevant restriction endonuclease cleavage sites;

Fig. 10 - λ Charon 30 Map of relevant restriction endonuclease cleavage sites;

Fig. 11 - λ L4 Map of relevant restriction endonuclease cleavage site, topological features, transcription from lux promoter, and transcription from P_L; and

Fig. 12 - λ L28 Map of relevant restriction endonuclease cleavage site topological features transcription from lux promoter, and transcription from P_L.

3. Specific Examples

The invention is further illustrated by the following Examples. These examples are to be considered as models only which demonstrate the general and broad principle of the invention as specified herein, and they are not to be understood or limiting the invention in any way.

Example 1 - Determination of Escherichia coli strain W3110 by Transduction with phage λL28.

E. coli W3110 cells were grown in liquid FT medium to early logarithmic phase of growth at 30°C. The culture was diluted tenfold and duplicate 1 ml samples were transferred to sterile vials. To each vial 25 μl of λ L28, $5x10^9$ PFU/ml (PFU=plaque forming units) was added. The λ L28 (ATCC No. 40183) was prepared as described above in Part XI of Materials and Methods. The vials were incubated at 25°C in the scintillation counter and the luminescence was determined as a function of time as described above in Part XIII of Materials and Methods. Fig. 1 shows the luminescence after 60 and 120 minutes of incubation as a function of E. coli concentration. The background count was 15,000 cpm.

Example 2 - <u>Determination of Escherichia coli strain W3110 by</u> <u>transduction with phage</u> λ <u>L4</u>.

5

<u>E</u>. <u>coli</u> cells were grown in liquid FT medium. The culture was diluted in saline (100 ml) to give final cell concentrations of 10^1, 10^2, 10^3 and 10^4 cells/ml. 100 ml of each sample was filtered through 0.45 micron Millipore filters and the filters were placed face upward in sterile scintillation vials. To each vial was added 1 ml of liquid medium containing λL4 at 7 x 10^7 PFU/ml, the λ L4

10
having been prepared as described above in Part XI of Materials and Methods. The luminescence of the vials was determined after 40, 60 and 100 minutes of incubation at 22°C, as described above in Part XIII of Materials and Methods. Fig. 2 shows the relation between the light emission (CPM) and the concentration of <u>E</u>. <u>coli</u> cells.

15

Example 3 - <u>Determination of Escherichia coli strain CSH1 by trans-</u> <u>duction with</u> λ·cI857 plac5.

20

<u>Escherichia coli</u> strain CSH1 was grown with shaking in FT medium at 37°C. Numbers of cells per ml were determined by viable counts on LB plates after a 20 hr incubation at 37°C. Expotentially growing cells were doubly diluted (dilutions of 2) with FT sterile medium. To each vial, isopropyl thiogalactoside (Sigma catalogue number

25
I5502) was added to give a final concentration of 100 mM.

λ cI857 S7 plac5 bacteriophage was added to give a final concentration of 10^9 plaque forming units/ml. Final volume was 2 ml. The vials were incubated with shaking at 37°C for 45 min. Then 2 ml of Z buffer (Miller, <u>op</u>. <u>cit</u>.) were added followed by the

30
addition of 0.1 ml chloroform and 0.05ml of SDS (0.1%). The contents of the vial were mixed with the aid of a Vortex mixer (30 sec) and 0.8 ml of ONPG (8 mg/ml) were added to each vial. ONPG was made up in 0.1M sodium phosphate buffer, pH 7.0. The vials were incubated with slight shaking at 37°C for 60 min. and the reaction

35
was stopped by the addition of 2 ml of Na_2CO_3 (1M). The developed color was determined with a Bausch and Lomb Spectronic 20 at 420nm

(for o-nitrophenol) and at 550nm for turbidity caused by cellular matter.

Figure 8 shows the amount of beta-galactosidase formed as determined by enzymatic assay as a function of cell concentration.

Example 4 - <u>Determination of Escherichia coli K strain MM294 by transformation with pBTK5.</u>

Recombinant DNA pBTK5 was prepared from the luminescence system fragment of <u>Vibrio fischeri</u> DNA and plasmid pBR322 as described above in Part IX of Materials and Methods. 1 μg of pBTK5 DNA was used to transform different concentrations of <u>E. coli</u> K strain MM294 in 0.2 ml of $CaCl_2$. After transformation, the cells were put in a scintillation vial containing 0.8 ml of LB and 0.2 ml of LB medium containing autoinducer prepared in accordance with Part XIV of Materials and Methods. Cell concentrations in different vials were 10^5, 10^6, 10^7, and 10^8 cells/ml. Fig. 3 shows the kinetics of light emission for the four different concentrations of <u>E. coli</u> cells and Fig. 4 shows the correlation between the cell concentration and the onset of light emissions in these experiments. The background light emission was 1000 cpm.

Example 5 - <u>Transformation of Escherichia coli strain MM294 in the presence of various antibiotics.</u>

Transformation was performed as in Example 3 and the transformed bacteria were subsequently incubated in the presence of various antibiotics. Ampicillin (AMP), 30 μg/ml; chloramphenicol (CAP), 10 μg/ml; kanamycin (KAN), 30 μg/ml and tetracycline (TET), 10 μg/ml. Fig. 5 shows the kinetics of luminescence with and without the antibiotics. The background count emission was 1000 cpm.

Example 6 - <u>Transformation of various bacteria with pBTK5 DNA.</u>

Transformation was performed as in Example 3, using as would be recipients Escherichia coli strain MM294, Aerobacter aerogenes strain 62-1, Proteus vulgaris and Staphylococcus albus. Fig. 6 shows the level of light emitted (cpm) as a function of time, and it

5   is seen therefrom that only E. coli was transformed while the remaining three species remain unaffected. This demonstrates the specificity of transformation with the recombinant DNA pBTK5. The background count was 1000 cpm.

10   Example 7 - <u>Determination of Escherichia coli strain W3110 by</u> <u>conjunction</u>.

The HfrH (high frequency of conjugation) strain containing the luminescence gene was constructed as described in XII (Materials and

15   Methods). This strain was grown in LB medium containing streptomycin (2.5 μ g/ml) for 2 hrs. at 37°C. The cells were washed once and diluted into LB medium to give a final cell density of 10^7 cells/ml. To 1 ml aliquots of this culture, different concentrations of (non-luminescent) E. coli strain W3110 cells were

20   added. The mixed cultures were incubated without shaking at 37°C for 30 minutes and then the cultures were taken for luminescence determinations in a scintillation counter at 24°C. Fig. 7 shows the luminescence developed in the presence of different concentrations

25   of E. coli W3110 recipient cells as a function of time (from time zero).

Example 8 - <u>General method for the isolation, cloning, and transfer</u> <u>of exogenous genes into microbial host organisms, and detection of the</u>

30   <u>recipient host using the expression of the introduced donor gene systems</u>.

As described previously herein; alternative enzyme or other systems in addition to luciferase and β -galactosidase can be introduced into host organisms for detection purposes in accordance with the

35   invention. The system or enzyme can be chosen because of the ease with which its presence may be determined or because it is unique

and absent in the organisms to be ascertained. The luciferase system previously described has both these qualities and is a preferred embodiment of the invention. Genes encoding proteins whose presence can be directly detected by their absorption of light at certain wavelengths (e.g., hemoglobin) or by specific antibodies, radioactively labelled or linked to conveniently determined enzymes (ELISA) or to other compounds such as biotin, can be used for the detection of microorganisms in the ways analogous to detection of luciferase. In other cases, genes encoding enzymes whose product or products can be measured directly or indirectly or in which the disappearance of one of its substrates can be similarly measured may be more suitable. Examples of such enzymes are given below in several Examples. Measurements might be based on evolution of gas or the reduction of the concentration of a dissolved gas, changes in pH, products or substrates that can be directly measured by spectrophotometry or chromatography or indirectly by coupled re-actions or by antibody detection of a secondary complex (e.g., biotin-avidin) or through the use of radioactively labelled com-pounds. Such measurements for identification purposes are given by means of illustration only and are not intended to limit the scope of the present invention, but to indicate the types of systems that might be used in conjunction with our method. Representative enzymes, proteins or enzyme systems can be utilized for detection of microorganisms after the relevant genetic structures have been assembled. Such assembly may involve natural recombination events or in vitro recombinant DNA techniques or both. There are numerous possibilities and strategies consistent with the present invention.

Various isolation, cloning, and transfer procedures involving foreign genes and introduction to host organisms can advantageously be used in the invention. For example, the DNA of donor organisms (i.e., bacterium, yeast, etc.) can be isolated and subjected to partial digestion by the restriction endonuclease Sau3A to yield a set of overlapping fragments encompassing the whole donor genome. Fragments of a particular size may be prepared by centrifugation

through glycerol or sucrose gradients or by agarose gel electrophoresis. These fragments contain protruding 5' single strand ends with the structure 5'GATC-double stranded DNA and can then be ligated by T4 DNA ligase to appropriate vector molecules cut

5   with enzymes (BamHI, BglII, BclI Sau3A) that created appropriate and compatible single strand ends. An example of such a vector is λCharon 28 (Maniatis et al., op. cit.) cut with restriction endonuclease BamHI. The ligated DNA can then be transformed, subsequent to packaging if desired, into an appropriate host (lack-

10   ing a restriction system). Viral DNA containing sequences for the desired exogenous system or enzyme can be detected either through enzymatic activity, immunological techniques or by synthetic or natural DNA probes and nucleic acid hybridization. Many of the above techniques of cloning and detection are detailed in Maniatis

15   et al. (op. cit.). Subsequently, the donor gene is subcloned and then connected to a powerful bacterial promoter (e.g., plac of Escherichia coli or P_R of bacteriophage λ). Then the promoter-gene segment can be transferred by recombination, natural, in vitro or both, to a conjugative plasmid (e.g., F factor or RP4),

20   a transformable element (e.g., plasmid pBR322) or to an appropriate bacteriophage (e.g., λ or T4).

The expressed donor gene or gene system thus transferred to a

25   receptive host microorganism can then be used to identify the host organism. A suitable method for monitoring the activity of the donor genetic system in the host organism is selected (i.e., light production with luciferase; end product or intermediate of enzymatic reaction; gas evolution, etc.). Thus, the isolation, cloning, and

30   transfer of foreign genes from a donor organism into a receptive host microorganism and identification of the host microorganism by monitoring expression of the foreign gene system contained therein is effected.

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Example 9 - <u>Method for the use of alcohol dehydrogenase or other NAD</u>
<u>oxidoreductases gene systems obtained from a foreign donor and trans-</u>
<u>ferred to a receptive host microorganism for detection of the host.</u>

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Alcohol dehydrogenase (E.C. 1.1.1.1; Alcohol:NAD oxidoreductase) is
an enzyme that is formed by many species such as yeast, man and
<u>Bacillus</u>. The gene or genes coding for this enzyme can be isolated
by recombinant DNA techniques. Using the method of Example 8, the
DNA of yeast as a donor organism is isolated, treated by restriction

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endonuclease, cloned, connected to an effective bacterial promotor,
and transferred by an appropriate vector, preferably a
bacteriophage, to a receptive host microorganism.

The ADH gene can then be used to detect the presence of specific

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microorganisms by techniques and strategy similar to those presented
earlier for bacterial luciferase and beta-galactosidase. In the
case of ADH, light production will not be directly monitored; rather
the conversion of NAD^+ to NADH would be measured. In the presence
of ethanol and NAD^+, ADH catalyses a reaction whose products are

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NADH, H^+ and acetaldehyde. The presence and concentration of NADH
can be determined by adsorbance of light at 340 nm or by bacterial
luciferase in the presence of an appropriate aldehyde (e.g., C_{14}
aldehyde) (Stanley, P.E. in Methods in Enzymology, volume LVII

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edited by M.A. DeLuca, Academic Press 1978, New York, San Francisco,
London, pp. 215-222).

There are many other NAD oxidoreductases that would be as suitable
as ADH (enzymes of E.C. classes 1.1.1, 1.2.1, 1.3.1, 1.4.1, 1.5.1

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and 1.8.1) for identification of host microorganisms. For example,
L-alanine dehydrogenase (E.C.1.4.1.1., L-alanine: NAD
oxidoreductase) also yields NADH from NAD^+. In addition, this
enzyme liberates NH_3 from L-alanine when it is converted to pyruvate
and NH_3 may be the product measured rather than NADH. Other

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oxidoreductases use O_2 as the electron acceptor in place of NAD^+
(E.C. classes 1.1.3, 1.2.3, 1.3.3 and 1.4.3) and the disappearance

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of dissolved oxygen might be followed by, for example, an oxygen electrode.

Example 10 - Method for the use of genetic systems coating for
transferase obtained from a foreign donor and transferred to a receptive
host microorganism for detection of the host.

Transferase enzyme systems can also be used to identify micro-
organisms in conjunction with the invention herein described. Using
the method of Example 8, the DNA of a suitable donor organism
containing the selected transferase gene(s) is isolated, treated by
restriction endonucleases, cloned, connected to an effective bacte-
rial promotor, and transferred by an appropriate vector (preferably
a bacteriophage), to a receptive host microorganism. For example,
L-tyrosine:2-oxoglutarate animotransferase (E.C.2.6.1.5) yields
p-hydroxyphenylpyruvate from L-tyrosine in the presence of
2-oxoglutarate and the appearance of p-hydroxyphenylpyruvate can be
monitored spectrophotometrically at 330 nm (Hadar, R., Slonim, A.,
and Kuhn, J., 1976. Role of D-tryptophan oxidase in D-tryptophan
utilization by Escherichia coli. J. Bacteriol. 125:1096-1104). A
second example of a transferase is galactokinase (ATP:
   -D-galactose-1-phosphotransferase; E.C.2.7.1.6) whose activity
can be followed by disappearance of ATP or appearance of
   β-D-galactose-1-phosphate. Other detection modes can be readily
adapted to the particular selected transferase.

Example 11 - Method for the use of hydrolase, lyase, or isomerase
genetic systems obtained from a foreign donor and transferred to a
receptive host microorganism for detection of the host.

In addition to the enzyme systems previously described, hydrolase,
lyase, and isomerase enzyme systems can be used to identify microor-
ganisms in conjunction with the present invention. Using the method
of Example 8, the DNA of a suitable donor organism containing the
selected enzyme (hydrolase, lyase, or isomerase) is isolated,

treated by restriction endonucleases, cloned, connected to an
effective bacterial promotor, and transferred by an appropriate
vector (preferably a bacteriophage), to a receptive host microorga-
nism. For example, alkaline phosphatase (E.C.3.1.3.1,
orthophosphoric monoester phosphohydrolase) activity can be de-
termined in a number of ways, one of which is by release of
p-nitrophenol from p-nitrophenylphosphate. p-nitrophenol concen-
tration can be determined spectrophotometrically at 420 nm at
alkaline pH in the same way that o-nitrophenol was used for deter-
mination of β-galactosidase (E.C.3.2.1.23) activity. Lyases such
as aspartate-1-decarboxylase (E.C.4.1.1.11, L-aspartate
1-carboxylyase) and ornithine decarboxylase (E.C.4.1.1.17,
L-ornithine carboxylyase) are equally suitable since the liberation
of CO_2 (as H_2CO_3) can be determined by changes in pH. Isomerases
such as glutamate racemase (E.C.5.1.1.3) can also be employed. In
the case of glutamate racemase the conversion of D-glutamate to
L-glutamate can be followed by coupling the system to L-glutamate
oxidase following conventional protocols.

Example 12 - <u>Method for the use of oxidase genetic systems obtained
from a foreign donor and transferred to a receptive host microorganism
for detection of the host.</u>

The introduction of enzymatic activities into the target host
microorganism wherein such enzymatic activity is either low or
non-existent. By use of the method of Example 8, the DNA of a
suitable donor organism containing the selected enzyme (i.e.,
oxidase) is isolated, treated by restriction endonucleases, cloned,
connected to an effective bacterial promotor, and transferred by an
appropriate vector (preferably a bacteriophage imparting
specificity) to a receptive host microorganism. One possible use
for the present invention involves, for example, D-tryptophan
oxidase (Hadar <u>et</u> <u>al</u>., <u>op</u> <u>cit</u>.) which is almost non-existent in
<u>Escherichia</u> <u>coli</u>. The introduction of the mutant gene leading to
high levels of this enzymatic activity is detectable through the

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production of indolepyruvic acid from D-tryptophan. Likewise elements containing the bio B gene of E. coli joined to strong promotor lead to rapid conversion of dethiobiotin to biotin, the latter of which can be detected by methods based on the high affinity of avidin for biotin.

4. Results

A. Transduction

Bacteriophage propagate themselves by adsorption and subsequent injection of their DNA into an appropriate bacterial host. In nature bacteriophage genes can sometimes become linked to bacterial genes through substitution and/or addition. Occasionally, some bacteriophage envelope accidently DNA coming from a source other than the bacteriophage (i.e. with chromosomal, plasmid or other bacteriophage species). The former is called specialized transduction, the latter generalized transduction. Both of these phenomena lead to the transfer of DNA from one bacterium to another.

A more modern way to produce specialized transducing phages is through the use of recombinant DNA technology. Phages can be constructed that carry desired genes and in which no essential bacteriophage genes have been lost. Such bacteriophages will efficiently introduce these cloned genes if an appropriate host is present. If elements for their expression exist, these genes will be highly expressed since many copies of the bacteriophage chromosome are made during the growth of the phage.

In accordance with a preferred embodiment of the present invention, the fragment of DNA carrying the luminescence genes of V. fischeri was transferred to the Escherichia coli bacteriophage λ from the plasmid pBTK5. Transference of

other donor genetic systems can be similarly effected by appropriate constructions.

Upon infection, the phage adsorb to the specific bacteria capable of acting as host, and the encapsulated DNA of the phage head is injected into the bacterial cell. Once the DNA is inside the bacterium it can make use of the host functions and it is transcribed and translated to produce luminescence enzymes (luciferase and those necessary for aldehyde synthesis) or other introduced enzyme systems.

The use of a luminescent system is a preferred embodiment of the invention. Since neither the phage, nor the bacteria emit light by themselves, the level of light subsequent to infection can be used to detect the presence of bacteria and the amount of light emitted reflects the number of phage-bacterium complexes. In the presence of excess phage the amount of light reflects the concentration of bacteria. Using this technique less than 10^4 Escherichia coli cells/ml can be detected within one hour (Fig. 1). When a sample containing bacteria was concentrated by filtration through Millipore HA 0.45 micron filters, concentrations as low as 10 cells/ml could be detected.

In the presence of an antibiotic to which the host cell is sensitive, the infecting phage cannot complete its lytic cycle and the amount of light emitted is reduced or no light is emitted. Antibiotics that block DNA, RNA or protein synthesis were tested and shown to lower the output of light. Antibiotics that affect cell wall synthesis or membrane integrity gave similar results. Thus, if a given type of bacterium is detected by infection with phage carrying luminescence genes, the susceptibility of the bacterial host to antibiotics can be rapidly determined by examining the effect of an antibiotic on light emission.

The presence of bacteria can also be detected by enzymatic means using transduction as illustrated in Figure 3. The presence of sufficient bacterial cells and the infection thereof by a bacteriophage carrying a gene coding for beta-galactosidase results in the de novo synthesis of beta-galactosidase whose presence and activity may be determined by enzymatic assay. The greater the number of infected complexes, the greater the amount of enzyme formed.

A similar experiment was performed using a λ bacteriophage carrying the gene for tryptophanase and a bacterial strain deficient for this activity. Again the presence of these bacteria led to enzyme formation, albeit at relatively high concentrations of cells (10^9 cells/ml).

Bacteriophage generally can only grow on some or all strains within a given single species of bacteria. Occasionally a given type of bacteriophage can infect several species but these are almost always found to be closely related. By choosing appropriate bacteriophage types, the test for bacteria can be made as specific as desired. This means that not only can the presence of bacteria be detected in this test but also the presence or absence of specific types of bacteria can be rapidly determined. For example, if the detection of Escherichia coli is desired, a mixture of phages whose host ranges span all known strains of this species and which carry luminescence genes, would allow the rapid specific detection of this species.

B.   Transformation

These experiments illustrate that bacteria can be detected by transforming them with a compatible DNA containing bioluminescence or other donor genes. The presence of bacteria, their approximate numbers, their taxonomic position

and their susceptibility to antibiotics can be determined through this technique.

When _Escherichia_ _coli_ strains JM101 and MM294 were transformed with plasmids pBTK5 and pAChv-1 obtained, respectively, according to parts IX and X of Materials and Methods, demonstrable light was emitted within a few hours. Cells without added DNA or the DNA alone were totally dark. The level of light emitted was several thousand fold that of the background.

In a separate experiment the amount of light emitted was measured when the added DNA is constant (1 µg) and the cell number varied. The results are shown in Fig. 3 and illustrate that the time of onset of emission is negatively correlated with cell concentration. This is clearly shown in Fig. 4 in which the time of onset of light emission is plotted against cell concentration. The almost linear relationship obtained demonstrates that this method can also be used to approximate cell concentration.

Another application of transformation using light producing genes is in the determination of the susceptibility of bacteria to antibiotics. The plasmid used was pBTK5 (1 µg) and strain MM294. After transformation antibiotics were added and their effect on light emission determined. The experimental controls were $CaCl_2$ treated cells without added plasmid DNA and transformed cells without added antibiotics. Fig. 5 shows the experimental results obtained. Kanamycin (30 µg/ml) and chloramphenicol (30 µg/ml) entirely prevented the production of light. Tetracycline (10 µg/ml) caused a 90% decrease versus that of the control without antibiotic. Ampicillin (30 µg/ml) actually stimulates light production. That ampicillin would not inhibit or only slightly inhibit light emission was expected because pBTK5 caries a determinant for ampicillin resistance. The unexpected stimulation may be due

to selection for increased replication of the entering plasmid or to increased transcription of the light genes via the bla· gene (ampicillin resistance) promoter. Thus the sensitivity of a bacterial population to an antibiotic (chloramphenicol,

5   tetracycline and kanamycin in these experiments) can be rapidly determined through transformation with bioluminescence genes. Resistance (ampicillin) is also rapidly detected. Furthermore, the activity of an antibiotic can be rapidly ascertained as a function of its concentration (tetracycline versus

10   chloramphenicol and kanamycin). A suboptimal concentration or partial resistance is also detected by this test (tetracycline).

An additional feature of transformation is its specificity. As

15   shown above, two strains of Escherichia coli were readily detected. When an experiment was conducted in which Escherichia coli, Proteus vulgaris, Staphylococcus albus and Aerobacter aerogenes were transformed by pBTK5 DNA, only Escherichia coli emitted light (Fig. 6). Since Aerobacter and

20   Proteus are quite closely related to Escherichia coli, this shows that this test has a good deal of specificity.

C.   Conjugation

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Certain bacteria have the capacity to donate their DNA to other cells (recipients). This process, known as conjugation, is mediated by plasmids which are circular DNA molecules whose presence is often superfluous to the cell's health. Not all

30   plasmids are able to promote conjugation: only those that have transfer genes are able. When the plasmid exists as a free-non-integrated molecule it usually mediates its own transfer from donor to recipient and only occasionally the transfer of other plasmids or of the bacterial chromosome.

35   However, when a conjugating plasmid is integrated into the chromosomes, it primarily transfers the chromosome and only

rarely succeeds in transferring itself. The range of possible recipients depends on the conjugating plasmid itself. For example, plasmid RP4 can transfer itself between quite distantly related gram negative bacterial while F^+ can transfer itself to many fewer, closely related types.

Escherichia coli K12 strain AT2446 that is an Hfr was made doubly lysogenic as described in Part XII of Material and Methods. AT2446 is an HfrH strain and hence transfers its DNA to a recipient starting from 0 minutes. The donor DNA is transferred clockwise with thr (Bachmann, B. J. and K. B. Low, 1980: Linkage map of Escherichia coli K12, edition 6. Microbiol. Rev. 44:1-56) the first bacterial chromosomal marker and malB at 91 minutes about the last. Since the attachment site of bacteriophage λ is at 17 minutes, HfrH should transfer the lysogenic virus (in this case viruses) fairly early and at a high efficiency. When transfer of λ to a non-lysogenic strain occurs, zygotic induction will take place. This phenomenon is the result of the viral genome entering a cytoplasm that lacks the repressor of λ . Without repressor genes, genes begin to express themselves, the phage excises itself from the bacterial chromosome, and the lytic cycle proceeds. This gives rise to approximately 300 genomes of λ per cell and the light genes will be expressed at high levels because of their increased dosage (amount) and because they will also be highly transcribed by the λ promoter P_L which was previously repressed.

Some background light emission will occur in these strains since the luminescence genes will be expressed from their own promoter. We have discovered that the background light can be greatly suppressed by growing the donor strains with sublethal concentrations of streptomycin. It was found that streptomycin selectively inhibits the induction of the luminescent system while almost not affecting the rate of growth.

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An experiment illustrating the use of conjugation and
luminescence is described in Example 6 and as shown in Fig. 7,
the amount of light emitted is directly related to the concen-
tration of the recipient cells at a given donor concentration.
That the relation between emitted light is linearly related to
recipient concentration is illustrated in the insert of Fig. 7.
Antibiotics like chloramphenicol (10 μ g/ml) and streptomycin
(100 μ g/ml) totally inhibited the increase in emitted light
when they were included during mating.

Using this technique it is possible to determine the presence,
the number and the sensitivity to antibiotics of bacteria in a
sample. Conjugation is not limited to Hfr's or lysogenic
bacteriophages. Any strain can be used which contains a
luminescence system, or parts thereof, or other donor genetic
system which is absent or poorly expressed in the host
organism, in a transferable replicon. The replicon may be the
bacterial chromosome or a plasmid. The luminescence system or
parts thereof, or other genetic system may be introduced by a
bacteriophage or by genetic recombination, either natural or
artificial, such that the luminescence or other genes are
covalently linked to the transferred genetic material. Thus
conjugation is analagous to the use of light coupled
transduction and transformation described above.

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WHAT IS CLAIMED IS:

1. A method for determining a specific type or group of microorganisms in a milieu comprising the steps of:

(a) transferring by genetic means biologically-functional natural or recombinant DNA encoding for a genetic system, or operative part thereof, which is derived from a eukaryotic or prokaryotic donor source into a receptive microbial host, wherein said introduced donor genetic system is normally absent or poorly expressed by said host; and

(b) detecting the expression of said donor genetic system by said host microorganism by physical, chemical, or biological means; and thereby

(c) identifying the presence of said host microorganism.

2. A method according to Claim 1 wherein said donated genetic system is a natural or recombinant DNA expressing a luciferase or other luminescent system, beta-galactosidase, alcohol dehydrogenase or other NAD oxidoreductase, transferase, alkaline phosphatase or other hydrolase, lyase, isomerase, or D-tryptophan oxidase or other oxidase, and the donor genetic system is transferred by conjugation, transformation, or transduction, and said host microorganism is selected from the genera consisting of Escherichia, Aerobacter, Salmonella, Shigella, Klebsiella, Proteus, Pseudomonas, Staphylococcus, Streptococcus, Chlamydia, Mycoplasma, Pneumococcus, Neisseria, Clostridium, Bacillus, Corynebacterium, Mycobacterium, Camphybacter, Vibrio, Serratia, Enterobacter, Providencia, Chromobacterium, Brucella, Yersinia, Haemophilus, and Bordetella.

3. A method according to Claim 2 wherein said donor genetic system comprises a luciferase or other luminescent system, or functional parts

thereof, derived from a biological source, said host is a bacterium, and wherein said luminescent donor system is detected in said host bacterium by the emission of light, and wherein said genetic system is transferred by transformation or transduction using plasmid or bacteriophage vectors.

4. A method according to Claim 3 wherein said luminescent system is a luciferase system derived from <u>Vibrio fischeri</u> or other microbial source.

5. A method according to Claim 4 wherein said luminescent system comprises recombinant DNA attached to an expression control sequence capable of effecting the expression of said luminescent system in said host bacterium.

6. A method according to Claim 5 wherein transfer of said luminescent system involves transduction and said vector is a bacteriophage comprising recombinant DNA packaged in a capsid and said bacteriophage recognizes said host bacterium or strains thereof or similar bacterial group related thereto.

7. A method according to Claim 6 wherein said bacteriophage is L28 (ATTCC No.40183), or λL4 or functional mutants thereof.

8. A method according to Claim 5 wherein transfer of said luminescent system involves transformation and said vector is a plasmid.

9. A method according to Claim 8 wherein said transfer vector is pBTK5 or pAChv-1.

10. A method according to any one of Claim 3, 4, 5, 6, 7, 8, and 9 wherein said DNA transfer is performed in the presence of autoinducer of the luminescent system or functional parts thereof.

11. A method according to Claim 10 wherein an aldehyde is added to the milieu in which said DNA transfer is carried out.

12. A method according to Claim 5 wherein a transposon is used to construct a bacteriophage containing all or part of the luminescent system.

5   13. A method according to Claim 2 wherein said donor DNA transfer is effected by conjugation by means of bacterial strains containing a donor genetic system or functional parts thereof in a transferable replicon, being a bacterial chromosome or a plasmid.

10   14. A method according to Claim 13 wherein the donor DNA system or functional parts thereof is introduced into said bacterial strain by a bacteriophage.

15. A method according to Claim 13 wherein said donor system is a
15   luminescent system or functional parts thereof which is introduced into said bacterial strain by natural or artificial genetic recombination.

16. A method according to Claim 13 wherein bacterial strains are used that are Hfr's which are lysogenized by temperate bacteriophages
20   carrying the donor DNA system.

17. A method according to any one of the preceeding Claims wherein said DNA transfer is performed in the presence of an antibacterial agent
25   to determine the susceptibility of the host organism to said antibacterial agent.

18. Recombinant bacteriophage transfer vector for use in conjunction with the method of Claim 1 prepared by the steps comprising:
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        (a) selecting a host microorganism or combinations thereof
             from the group of Escherichia, Aerobacter, Salmonella,
             Shigella,   Klebsiella,   Proteus,   Pseudomonas,
             Staphylococcus, Streptococcus, Chlamydia, Mycoplasma,
35   Pneumococcus,   Neisseria,   Clostridium,   Bacillus,
             Corynebacterium, Mycobacterium, Camphybacter, Vibrio,

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Serratia, Enterobacter, Providencia, Chromobacterium, Brucella, Yersinia, Haemophilus, and Bordetella;

(b) obtaining an essentially biologically-pure bacteriophage which is specific for the host selected in step (a) above;

(c) selecting a natural or recombinant DNA genetic system from a eukaryotic or prokaryotic donor source which is absent or poorly-expressed in the selected host microorganism;

(d) attaching an expression control sequence to said donor DNA system which is capable of effecting the expression of said donor system in the selected host microorganism; and

(e) transforming, with or without packaging, said biologically-functional DNA prepared in step (d) to yield a bacteriophage as selected in step (b) above containing a recombinant genetic system.

19. A recombinant bacteriophage according to Claim 18 wherein said donor DNA system expresses a luciferase or other luminescent system, beta-galactosidase, alcohol dehydrogenase or other NAD oxidoreductase, transferase, alkaline phosphatase or other hydrolase, lyase, isomerase, or D-tryptophan oxidase or other oxidase.

20. A recombinant bacteriophage according to Claim 19 wherein said donor DNA system is a luciferase or other luminescent system.

21. A biologically-pure culture of recombinant bacteriophage λ 28 according to Claim 20 which carries a luminescent genetic system derived from Vibrio fischeri and having Escherichia coli as its host and having the identifying characteristics of ATCC 40183.

22. A method for replicating in a microbial system a biologically functional protein encoded by a DNA sequence for the transcription and translation of luciferase comprising the steps of:

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      (a)   extracting mRNA coding for luciferase from a eukaryotic or prokaryotic donor;

      (b)   isolating and purifying the mRNA coding for luciferase obtained from said donor;

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      (c)   synthesizing a single-stranded cDNA having a nucleotide sequence complimentary to that of the isolated mRNA with reverse transcriptase;

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      (d)   synthesizing double-stranded cDNA with the second strand being complimentary to the single-stranded cDNA synthesized above, using DNA polymerase;

      (e)   treating the double-stranded cDNA enzymatically to provide

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      end groups which are suitable for insertion of said cDNA into a DNA cloning vector;

      (f)   linearizing a transfer vector and inserting said cDNA into

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      said vector and ligating said vector forming a recombinant cloning vector;

      (g)   transforming a microorganism with said recombinant plasmid cloning vector;

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      (h)   isolating a strain of said transformed microorganism containing said recombinant cloning vector containing a cDNA sequence coding for luciferase;

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      (i)   enzymatic treating said cloning vector enzymatically so as to provide DNA fragments encoding for luciferase or

portions thereof which are suitable for insertion into a
DNA expression vector;

(j)   inserting said DNA fragments encoding for luciferase into
an expression vector characterized by an expression
control group which effects the expression of genes of
prokaryotic or eukaryotic cells and their viruses; and

(k)   transferring said expression vector into a host
microorganism whereupon said host expresses said cDNA
thereby replicating biologically-functional luciferase.

23. A method according to Claim 22 wherein said expression vector
expresses cDNA and thereby synthesizes luciferase and the subsequent
emmission of light is used to detect said host microorganism and to
determine sensitivity of said microorganism to antibiotics.

24. A method according to Claim 22 wherein said recombinant
expression vector is a plasmid.

25. A method according to Claim 22 wherein said recombinant
expression vector is a bacteriophage.

26. Recombinant plasmid transfer vector for use in conjunction with
the method of Claim 1 prepared by the steps comprising:

(a)   selecting a host microorganism or combinations thereof
from the group consisting of Escherichia, Aerobacter,
Salmonella, Shigella, Klebsiella, Proteus, Pseudomonas,
Staphylococcus, Streptococcus, Chlamydia, Mycoplasma,
Pneumococcus, Neisseria, Clostridium, Bacillus,
Corynebacterium, Mycobacterium, Camphybacter, Vibrio,
Serratia, Enterobacter, Providencia, Chromobacterium,
Brucella, Yersinia, Haemophilus, and Bordetella;

(b) selecting natural or recombinant DNA encoding for a donor genetic system, or operative part thereof, which is derived from a eukaryotic or prokaryotic donor source and said donor genetic system is normally absent from or poorly expressed by said host microorganism;

(c) enzymatic treatment of said donor DNA to provide end groups suitable for insertion of said DNA into a cloning vector;

(d) selecting a plasmid vector compatible with said selected host microorganism;

(e) linearizing said plasmid vector;

(f) inserting said donor DNA into said plasmid vector and ligating said vector forming a recombinant cloning vector;

(g) transforming said host microorganism with said recombinant cloning vector;

(h) isolating a transformant containing said recombinant cloning vector;

(i) enzymatic treatment of said cloning vector so as to provide DNA fragments encoding for said donor genetic system or portions thereof which are suitable for insertion into a DNA plasmid expression vector compatible with said host microorganism;

(j) inserting said treated DNA fragments into a plasmid expression vector characterized by an expression control group which effects the expression of said donor genetic system, thus obtaining a desired plasmid transfer vector.

27. A plasmid transfer vector according to Claim 26 wherein said donor genetic system or part thereof is a natural or recombinant DNA expressing a luciferase or other luminescent system, beta-galactosidase, alcohol dehydrogenase or other NAD oxidoreductase, transferase, alkaline
5   phosphatase or other hydrolase, lyase, isomerase, or D-tryptophan oxidase or other oxidase.

28. A plasmid transfer vector according to Claim 27 wherein said donor genetic system is a luciferase or other luminescent system.
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29. Recombinant plasmid pAChv-1 according to Claim 28 carrying a luminescent genetic system as described herein and as it occurs in the biologically-pure culture of E. coli MM294/pAChv-1 (ATCC 53133).

15   30. A method for determining the presence and concentrations of a specific antibiotic or mixtures thereof in a milieu comprising the steps of:

(a)   introducing   an   effective   concentration   of   an
20   antibiotic-sensitive assay bacterium into said milieu,
            said assay bacterium comprising natural or recombinant DNA
            encoding for a genetic system, or operative part thereof,
            derived from a eukaryotic or prokaryotic donor source, and
25   said biologically-functional assay system was introduced
            by genetic means to said assay bacterium which lacked or
            poorly expressed said assay system prior to said
            introduction;

30   (b)   incubating said assay bacterium in said milieu for a
            period of time;

(c)   detecting the expression of said assay system by said
            assay bacterium in said milieu by physical, chemical or
35   biological means; and

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    (d)   comparing said measured expression of said assay system to known responses of said assay bacterium to antibiotics; and thereby

    (e)   identifying the presence and concentration of antibiotic or antibiotic mixture in said milieu.

31. A method according to Claim 30 wherein said introduced genetic system is natural or recombinant DNA expressing a luciferase system, beta-galactosidase, alcohol dehydrogenase or other NAD oxidoreductase, transrerase, alkaline phosphatase or other hydrolase, lyase, isomerase, or D-tryptopham oxidase or other oxidase, and the introduced genetic system is transferred to said assay bacterium by conjugation, transformation, or transduction.

32. A method according to Claim 31 wherein said assay bacterium is <u>Escherichia coli</u> and said introduced genetic system is a luciferase or beta-galactosidase system, and said antiotic is selected from the group of: pencillin, ampicillin or other drug that inhibits cell well synthesis; streptomycin, erythromycin, kamamysin, neomycin, gentamicin or other animoglycoside; isoniazid; tetracycline, chloramphemical or other broad-spectrum antibiotic; polymyxin; sulfone; and sulfonamide, or mixtures thereof.

33. A method according to Claim 32 wherein said genetic system is a luciferase system and said expression thereof is detected by measurement of light.

34. A method for replicating in a microbial host system a biologically-functional protein encoded by a DNA sequence for luciferase or portions thereof or other luminescent system comprising the steps of:

    (a)   extracting DNA encoding for luciferase or other luminescent system from a suitable eukaryotic or prokaryotic donor;

(b) isolating and purifying the DNA obtained from said donor;

(c) treating said donor DNA enzymatically to provide end groups suitable for insertion of said DNA into a cloning vector;

(d) inserting said donor DNA into a cloning vector and ligating said vector forming a recombinant cloning vector;

(e) transforming a microorganism with said recombinant cloning vector;

(f) treating said cloning vector enzymatically so as to provide DNA fragments encoding for luciferase or portions thereof or other luminescent system which are suitable for insertion into a DNA expression vector;

(g) inserting said DNA fragments into an expression vector characterized by an expression control group which effects the expression of genes of prokaryotic or eukaryotic cells and their viruses; and

(h) transferring said expression vector into a host microorganism which normally lacks or poorly expresses said donor luminescent system whereupon said microbial host expresses said donor DNA thereby replicating biologically-functional luciferase or other luminescent system.

35. A method according to Claim 34 wherein said expression vector expresses said DNA and thereby synthesizes luciferase and the subsequent emission of light is used to detect said host microorganism and to determine sensitivity of said microorganism to antibiotics.

36. A method according to Claim 34 wherein said recombinant expression vector is a plasmid or a bacteriophage.

37. A method for assaying compositions or mixtures thereof for antibiotic activity in a milieu comprising the steps of:

(a) introducing an effective concentration of an antibiotic-sensitive assay bacterium into said milieu, said assay bacterium comprising natural or recombinant DNA encoding for a genetic system, or operative part thereof, derived from a eukaryotic or prokaryotic donor source, and said biologically-functional assay system was introduced by genetic means to said assay bacterium which lacked or poorly expressed said assay system prior to said introduction;

(b) incubating said assay bacterium in said milieu for a period of time;

(c) detecting the expression of said assay system by said assay bacterium in said milieu by physical, chemical or biological means; and

(d) comparing said measured expression of said assay system to known responses of said assay bacterium to antibiotics and controls; and thereby

(e) identifying the presence and concentration of antibiotic or antibiotic mixture in said milieu.

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38. A method according to Claim 37 wherein said introduced genetic system is natural or recombinant DNA expressing a luciferase system, beta-galactosidase, alcohol dehydrogenase or other NAD oxidoreductase, transrerase, alkaline phosphatase or other hydrolase, lyase, isomerase,
5   or D-tryptopham oxidase or other oxidase, and the introduced genetic system is transferred to said assay bacterium by conjugation, transformation, or transduction.
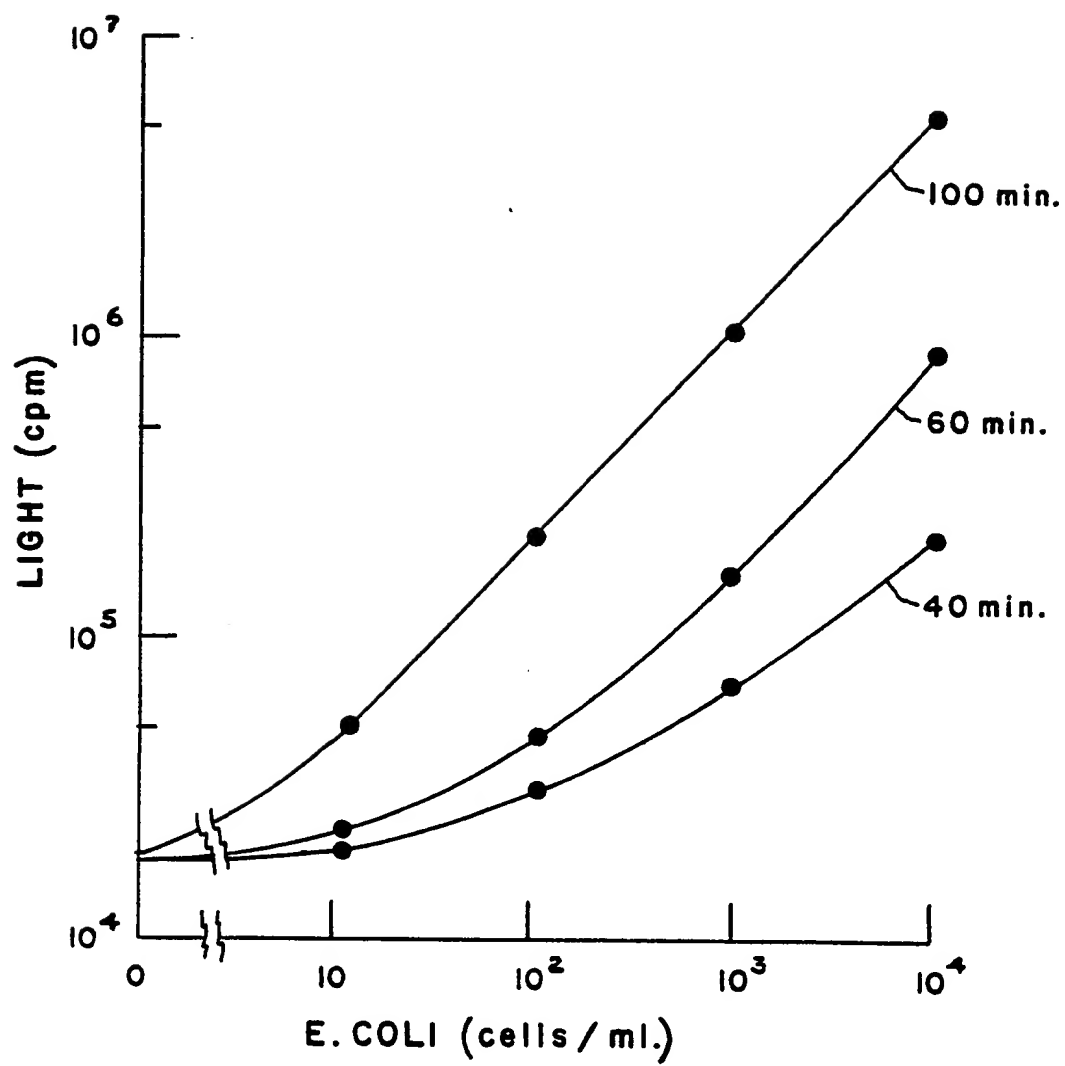
39. A method according to Claim 38 wherein said assay bacterium is
10   Escherichia coli and said introduced genetic system is a luciferase or beta-galactosidase system, and said antiotic is selected from the group of: pencillin, ampicillin or other drug that inhibits cell well synthesis; streptomycin, erythromycin, kamamysin, neomycin, gentamicin or other animoglycoside; isoniazid; tetracycline, chloramphemical or other
15   broad-spectrum antibiotic; polymyxin; sulfone; and sulfonamide, or mixtures thereof.

40. A method according to Claim 39 wherein said genetic system is a luciferase system and said expression thereof is detected by measurement
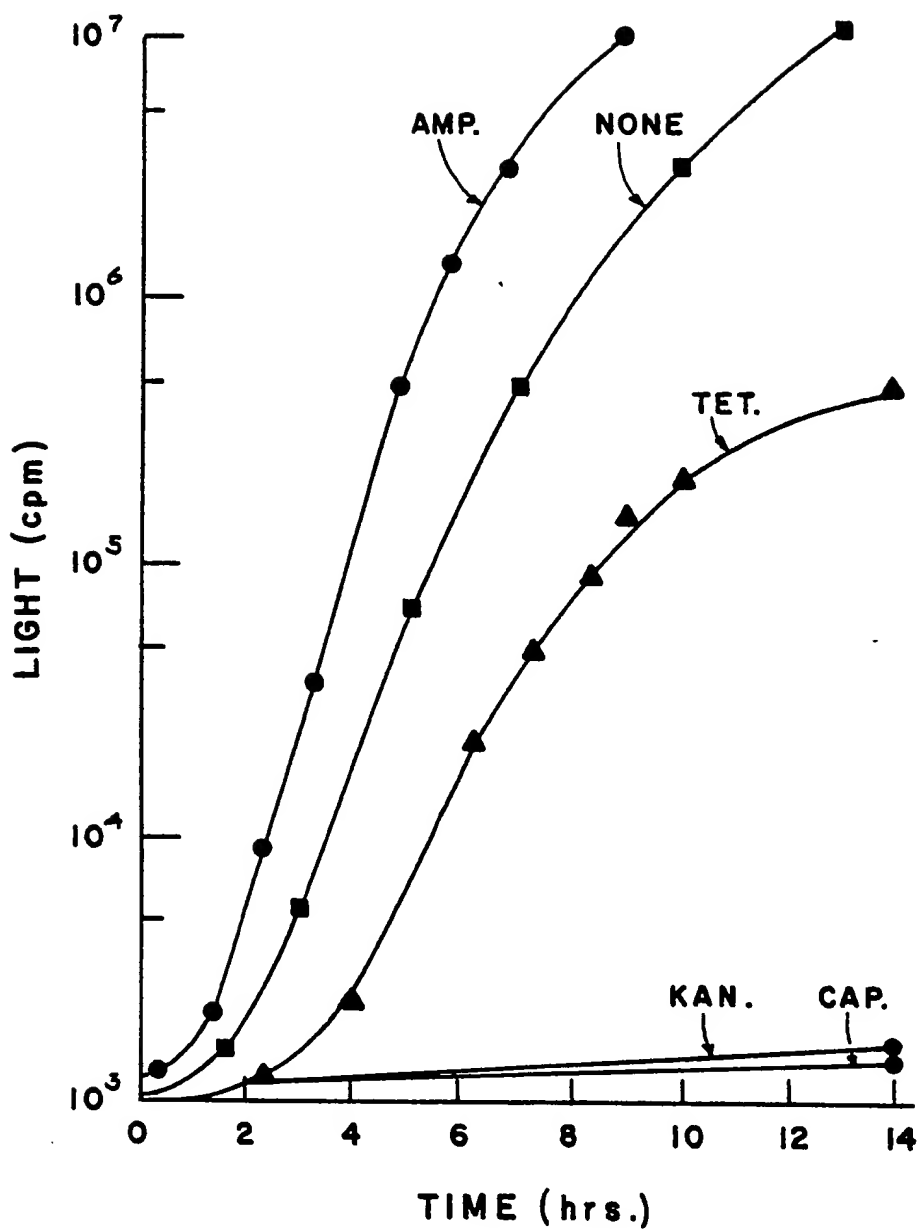20   of light.

25

30

35

Fig. I

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7 Sht. 1 of 2

Fig. 7

Sht. 2 of 2

Fig. 8

ScaI 16420
ScaI 18683

ScaI 25684
ScaI 27262

SalI 32745
ScaI 32801
SalI 33244

λ   Wild Type

Fig. 9

ScaI 16420
ScaI 18683
20285 PstI
22425 PstI
Deletion b1007   23616–28312
32009 PstI
32256 PstI
Sal I 32745
ScaI 32801
SalI 33244
34449
Duplication   22346–23316
Deletion b1007 23316–28312
Duplication   28312–34449
SalI 32745
ScaI 32801
SalI 33244
34449
Deletion K1154 35384–37925

Deletion nin5 40502–43307

λ   Charon 30

Fig. 10

0168933

λ   L4

Fig. II

λ L28

Fig. 12